PEPTIDE ANALOGUES AND USES THEREOF, IN PARTICULAR IN PHARMACEUTICAL AND DIAGNOSTIC COMPOSITIONS

The present invention relates to peptide analogues and to their uses, mainly in preparing pharmaceutical compositions, in particular vaccines, and for *in vitro* or *in vivo* diagnosis of various pathologies.

The development of neuropeptides, peptide hormones and peptide-based antibiotics, or peptide-based synthetic vaccines, is greatly disrupted by the high sensitivity of peptides towards proteolysis, which limits, inter alia, their oral and parenteral administration.

Pseudopeptides are a class of molecules which are particularly advantageous in the design of enzyme inhibitors. Cleavage with peptidases is involved in a variety of biological processes, since it makes it possible to generate active peptide or protein fragments from inactive precursors. The idea that an isosteric bond could be considered as a mimic of the tetrahedral intermediate formed during passage through the transition state has opened the way to inhibitors of pseudopeptide type. At the start of the 1980s this approach was used successfully in the development of powerful inhibitors of angiotensin conversion enzyme (ACE), of renin (Szelke *et al.*, 1982 ; Boger *et al.*, 1983) and of the aspartic protease of HIV (Huff, 1991). More recently, several selective pseudopeptide inhibitors of Ras farnesyl transferase have been proposed. These molecule are of potential therapeutic value in the treatment of cancers involving the mutated Ras oncogene (Buss & Marsters, 1995).

Pseudopeptidiques analogues of peptide hormones have also been the subject of many studies, with the prime objective of stabilizing the starting peptide and conserving the activity. This strategy has effectively allowed the discovery of powerful agonists (Chorev *et al.*, 1979 ; Nagain *et al.*, 1988 ; Doulut *et al.*, 1992), but also of partial agonists and even antagonists of the hormone studied (Martinez *et al.*, 1985 ; Coy *et al.*, 1989 ; Leban *et al.*, 1993 ; Cai *et al.*, 1994). Analogues which are more selective at the receptor level have also been obtained in certain cases (Mendre, 1988 ; Nagain *et al.*, 1988 ; Schiller *et al.*, 1993). Moreover, the pseudopeptide bond makes it possible to explore the conformational characteristics of the ligand at the modified site and to evaluate the contribution of the amide bond and the inter- or intramolecular hydrogen bonding in the interaction with the receptor or receptors.

However, the modifications made hitherto on peptides for immunological use are limited essentially to the addition of sugars, to that of longer or shorter amino acids to allow the emulsification of the resulting lipopeptides, to cyclization by formation of disulphide or covalent bridges, as well as to the addition of molecules such as biotin, in order to increase, for example, the sensitivity of solid-phase immunochemical tests.

Several authors have asserted that pseudopeptides probably have very little or no immunogenicity, since they could not be transformed and presented to the molecules of the major histocompatibility complex (MHC) in order to be recognized by the helper T cells or by the cytotoxic T lymphocytes. In this respect, Dintzis *et al.* have described that the L enantiomer of rubredoxin induces a strong immune response by production of immunoglobulins of isotype G (IgG), whereas the corresponding protein consisting of amino acids all of D configuration does not induce an immune response.

It is in this context that the immunoretroids described below, as well as the antibodies directed against these immunoretroids, have been described in International Patent Application WO 95/24916 as being useful in vaccinating against pathologies in which peptides from which they are derived (parent peptides) were involved, as well as for the diagnosis of these pathologies.

The authors of that International Patent Application, who are also the authors of the present application, had, in point of fact, demonstrated for the first time that the modification by replacing a peptide bond -CO-NH- in the peptide chain of a peptide, with a bond -NH-CO-, and, where appropriate, by the replacement of an amino acid of L configuration with an amino acid of D configuration, makes it possible to obtain peptide analogues (retro or retro-inverso, respectively) which can be used in the treatment of pathologies involving the immune response to humoral or cell mediation.

The abovementioned immunoretroids are compounds of the peptide type, i.e. compounds consisting of a chain of protein-generating amino acids, in which at least one of the -CO-NH- bonds, and advantageously all of the -CO-NH- bonds, in the peptide chain of the corresponding parent peptide (not comprising any -NH-CO- bonds in its peptide chain) is (are) replaced with one (or more) -NH-CO- bonds, the chirality of each aminoacyl residue, whether or not it is involved in one or more of the abovementioned -NH-CO bonds, being either conserved (retro peptide) or inverted (retro-inverso peptide) relative to the corresponding aminoacyl residues constituting the said parent peptide.

The expression "retro-inverso peptide" should be understood as meaning any peptide or peptide analogue corresponding to the definition of immunoretroids given above, the said peptide consisting more particularly of a peptide chain in which at

least one of the residues is linked to at least one neighbouring residue via an -NH-CO- bond, on the one hand, and has the opposite chirality to that of this same aminoacyl residue in the peptide chain of the parent peptide, on the other hand.

The expression "retro peptide" should be understood as meaning any peptide corresponding to the definition of immunoretroids given above, the said peptide consisting more particularly of a peptide chain in which at least one of the residues is linked to at least one neighbouring residue via an -NH-CO- bond, the chirality of all of the aminoacyl residues involved in at least one -NH-CO- bond being conserved relative to the corresponding residue in the peptide chain of the parent peptide.

It goes without saying that the -CO-NH- et -NH-CO- bonds must be taken into account in the text hereinabove and hereinbelow, in the sense of the parent peptide chain going from the amino-terminal (N-terminal) end to the carboxy-terminal (C-terminal) end.

Given the general context of uncertainty regarding the efficacy of the peptide analogues recalled above, the authors of this International patent application WO 95/24916 considered that this inversion of the direction of the amide bond between protein-generating amino acids, and/or the modification of the configuration of the protein-generating amino acids, represented the only possible modifications of the parent peptides in order to obtain peptide analogues with a longer half-life, and biological properties, in particular immunological properties, which are comparable with, or even higher than, those of the abovementioned parent peptides.

The present invention derives from the discovery made by the authors of the present patent application that, contrary to what they believed they had correctly deduced in the context of their previous invention on account of the general context recalled above, these modifications of retro or retro-inverso type of the peptide chain of the parent peptides do not represent the only possible modifications for obtaining peptide analogues of interest, at least not in the context of the diagnosis and/or treatment of pathologies involving the immune response to cell mediation.

The reason for this is that the authors of the present patent application have demonstrated that the modification of natural peptides involved in pathologies in which immunity to cell mediation is involved, and more particularly the cytotoxic T lymphocytes, by replacing peptide bonds -CO-NH- in the peptide chain of the said natural peptides with bonds other than the -NH-CO- bonds, and/or by replacing the protein-generating amino acids in the natural peptide with non-protein-generating amino acids, makes it possible to obtain peptide analogues of the said natural peptides which can be used with the abovementioned advantages in the context of

the treatment or diagnosis of the said pathologies.

The expression "protein-generating amino acid" means, in the text hereinabove and hereinbelow, any amino acid which is involved in the construction of a natural peptide or protein.

The expression "non-protein-generating amino acid" means, in contrast with the above definition, any amino acid which is not involved in the construction of a natural peptide or protein. The expression "non-protein-generating amino acid" more particularly refers to any amino acid in which the carbon bearing the side chain R, i.e. the group -CHR-, located between -CO- and -NH- in the natural peptide chain, is replaced with a unit which is not involved in the construction of a natural peptide or protein.

The object of the present invention is to provide pharmaceutical compositions, and more particularly vaccines, comprising peptide analogues which have a markedly longer half-life than that of natural peptides or natural proteins, or than the half-life of synthetic peptides which may or may not be derived from these natural proteins (these natural proteins, or peptides which may or may not be derived from these natural proteins, also being referred to in the text hereinbelow by the expression "parent peptides or proteins"), of which they are analogues, while at the same time having biological activity, and more particularly immunological activity, which is comparable to, or even higher than, that of the abovementioned parent peptides or proteins, or alternatively an activity which is different to or opposite that of the said parent peptides or proteins

Another object of the present invention is to provide methods for the *in vivo* diagnosis or alternatively for the *in vivo* evaluation of an individual's immune response capacity in the context of pathologies in which natural peptides or natural proteins (which may be exogenous or endogenous) are liable to be involved by binding to the molecules of the MHC, the peptide-MCH complexes thus obtained [lacuna] to the receptors located on the T cells and recognizing the said natural peptides or natural proteins in their form complexed with the said molecules of the MHC.

A further object of the invention is to provide compositions and kits for carrying out the abovementioned methods of *in vivo* diagnosis or evaluation of the immune response.

A further object of the present invention is to provide methods for the *in vitro* diagnosis of pathologies associated with the presence of endogenous or exogenous proteins in an individual's body, these methods being carried out with the aid of the peptide analogues as defined above, or with the aid of antibodies directed against the complexes between these antibodies and the molecules of the MHC, and having the advantage of giving higher-quality performance than the diagnostic methods

currently carried out with the aid of the parent peptides or proteins, or with the aid of antibodies directed against these parent peptides or proteins.

A further object of the present invention is to provide new kits for carrying out such methods of *in vitro* diagnosis.

The present invention relates to the use of peptide analogues of parent peptides, these parent peptides being derived, where applicable, from exogenous or endogenous proteins, the said parent peptides interacting with molecules of the MHC in the context of pathologies involving an immune response to cell mediation, in man or animals, the said analogues being characterized in that they correspond to the said parent peptides in which:

- at least one peptide bond -CO-NH- of the peptide chain is modified, with the exception of modifications of the retro or retro-inverso type, or
- at least one amino acid of the peptide chain is substituted with a non-protein-generating amino acid, or
- at least one peptide bond -CO-NH- of the peptide chain is modified and at least one amino acid of the said peptide chain is substituted with a non-protein-generating amino acid,

for the preparation of a medicinal product intended for preventing or treating the abovementioned pathologies.

More particularly, the invention relates to the abovementioned use of peptide analogues derived from parent peptides which interact with molecules of the MHC of category I, in the context of pathologies involving cytotoxic T lymphocytes.

Advantageously, the peptide analogues used in the context of the present invention, and more particularly those derived from parent peptides which interact with molecules of the MHC of category I, correspond to the said parent peptides in which at least one peptide bond -CO-NH- of the peptide chain is modified, with the exception of modifications of the retro or retro-inverso type.

As has already been seen previously, the abovementioned expression "parent peptide" should be understood as meaning:

- either a peptide which exists per se in the natural state, in particular in a microorganism or in a higher organism (in particular in the human body),
- or any peptide of immunological interest obtained by peptide synthesis, from protein-generating amino acids,
- or a peptide derived from a protein as exists in the natural state in the abovementioned organisms, in particular by fragmentation of the said protein (in particular with the aid of suitable proteinases, followed by purification of the peptide under consideration),

or by peptide synthesis (according to the methods conventionally used in this field),

- or a peptide derived from a protein as exists in the natural state, but whose immunological activity has been modified, conserved or optimized by replacing certain amino acids of the natural sequence with protein-generating amino acids, for example after screening a library of peptide analogues obtained by peptide synthesis.

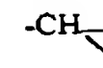
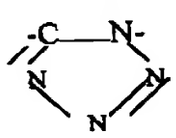
The invention also relates to the peptide analogues of parent peptides, these parent peptides being derived, where applicable, from exogenous or endogenous proteins, it being possible for the said parent peptides to interact with molecules of the MHC in the context of pathologies in man or animals, the said analogues being characterized in that they correspond to the said parent peptides in which :

- at least one peptide bond -CO-NH- of the peptide chain is modified, with the exception of modifications of the retro or retro-inverso type, or
- at least one amino acid of the peptide chain is substituted with a non-protein-generating amino acid, or
- at least one peptide bond -CO-NH- of the peptide chain is modified and at least one amino acid of the peptide chain is substituted with a non-protein-generating [lacuna] acid.

Advantageously, the abovementioned peptide analogues of the invention are characterized in that the number of protein-generating or non-protein-generating aminoacyl residues, linked via a modified or unmodified bond, is between about 5 and about 20, preferably between 8 and 12 for the peptide analogues which bind to the molecules of the MHC of category I, and preferably between 8 and 16 for the peptide analogues which bind to the molecules of the MHC of category II.

More particularly, the invention relates to the peptide analogues as described above, characterized in that at least one of the peptide bonds -CO-NH- in the peptide chain of the parent peptide is replaced with a bond other than the -CO-NH- bond, the said other bond being chosen in particular from the following :

$-CH_2-NH-$	(methyleneamino) ;
$-CH_2-CH_2-$	(carba) ;
$-CO-CH_2-$	(ketomethylene) ;
$-CH_2-O-$	(methyleneoxy) ;
$-CHOH-CH_2-$	(hydroxyethylene) ;
-CHOH-CHOH-	(dihydroxyethylene) ;
-CH=CH-	(E or Z olefin) ;
-CHCN-NH-	(cyanomethyleneamino) ;
$-S-CH_2-$	(thiomethylene) ;

$-CH_2-S-$	(methylenethio) ;
-CS-NH-	(thioamide) ;
$-PO_2-NH-$	(phosphonamide) ;
-CHOH-	(hydroxymethylene) ;
-NH-CO-NH-	(urea) ;
	(oxirane) ;
	(tetrazole) ;
$-CH_2-CO-NH-$	(β-homologation) ;
$-CHOH-CH_2-NH-$	(hydroxyethyleneamino) ;
-CO-NH-NH-	(hydrazino).

The invention also relates to the peptide analogues as described above, and characterized in that at least one of the peptide bonds -CO-NH- in the peptide chain of the parent peptide is replaced with a bond of the retro or retro-inverso type as defined above, in the case where at least one of the amino acids of the said peptide analogue is a non-protein-generating amino acid.

Peptide analogues which are preferred in the context of the present invention are characterized in that at least one of the peptide bonds -CO-NH- in the peptide chain or parent peptide is replaced with a methyleneamino bond or a bond of the β - homologation, or carba, or ketomethylene, or cyanomethyleneamino, or hydroxyethyleneamino type.

More particularly, the invention relates to the peptide analogues of parent peptides involved in melanoma, in particular the peptide MART1 27-35, the said parent peptide comprising, where appropriate, one or more mutations, such as the Leu^{28}-mutated parent peptide MART1 27-35, the said peptide analogues corresponding to the said parents peptides in which at least one of the peptide bonds -CO-NH- is modified, with the exception of modifications of the retro or retro-inverso type.

In this respect, the invention relates more particularly to the peptide analogues of the parent peptide Mart-1 27-35 of melanoma, comprising a methyleneamino bond and corresponding to the following formulae :

Sequences

	P1	P2	P3	P4	P5	P6	P7	P8	P9
MART1 27-35	H –A -	A -	G -	I -	G -	I -	L -	T -	V -OH
Ψ (1 – 2)	H -A $\Psi_{(CH_2NH)}$	A -	G –	I -	G -	I -	L -	T -	V -OH
Ψ (2 - 3)	H –A -	A $\Psi_{(CH_2NH)}$	G -	I -	G -	I -	L -	T -	V -OH
Ψ (3 – 4)	H –A -	A -	G $\Psi_{(CH_2NH)}$	I -	G -	I -	L -	T -	V -OH
Ψ (4 - 5)	H –A -	A	G -	I $\Psi_{(CH_2NH)}$	G -	I -	L -	T -	V -OH
Ψ (5 - 6)	H –A -	A	G -	I -	G $\Psi_{(CH_2NH)}$	I -	L -	T -	V -OH
Ψ (6 - 7)	H –A -	A	G -	I -	G	I $\Psi_{(CH_2NH)}$	L -	T -	V -OH
Ψ (7 - 8)	H –A -	A	G -	I -	G	I -	L $\Psi_{(CH_2NH)}$	T -	V -OH
Ψ (8 - 9)	H –A -	A	G -	I -	G	I -	L -	T $\Psi_{(CH_2NH)}$	V -OH

These peptide analogues ψ (1-2), ψ (2-3), ψ (3-4), ψ (4-5), ψ (5-6), ψ (6-7), ψ (7-8) and ψ (8-9), also referred to as reduced peptide analogues of Mart-1 27-35, correspond to the latter peptide in which the -CO-NH- bond located between the residues P1 and P2, P2 and P3, P3 and P4, P4 and P5, P5 and P6, P6 and P7, P7 and P8, and P8 and P9 is replaced, in each case, with a -CH_2-NH- bond.

The invention also relates to the peptide analogues of the parent peptide Mart-1 27-35 of melanoma, comprising a bond of the β -homologation type and corresponding to the following formulae :

Sequences

	P1	P2	P3	P4	P5	P6	P7	P8	P9
MARTI 27-35	H –A	A -	G -	I -	G -	I -	L -	T -	V -OH
β1	H -β-homoA	–A -	G -	I -	G -	I -	L -	T -	V -OH
β2	H -A	–β-homoA -	G -	I -	G -	I -	L -	T -	V -OH
β3	H –A -	A -β-homo	G -	I -	G -	I -	L -	T -	V -OH
β4	H –A -	A -	G -β-homo	I -	G -	I -	L -	T -	V -OH
β5	H –A -	A -	G -	I -β-homo	G -	I -	L -	T -	V -OH
β6	H –A -	A -	G -	I -	G -β-homo	I -	L -	T -	V -OH
β7	H –A -	A -	G -	I -	G -	I -β-homo	L -	T -	V -OH
β8	H –A -	A -	G -	I -	G -	I -	L -β-homo	T -	V –OH
β9	H –A -	A -	G -	I -	G -	I -	L -	T -β-homo	V –OH

These β1 to β9 peptide analogues of Mart-1 27-35 correspond to the latter peptide in which the -CO-NH- bond located between the residues P1 and P2, P2 and P3, P3 and P4, P4 and P5, P5 and P6, P6 and P7, P7 and P8, and P8 and P9, is replaced, in each case, with an $-CH_2-CO-NH-$ bond.

The invention also relates to the peptide analogues of the mutated parent peptide Mart-1 27-35 Leu^{28}, corresponding to the peptide Mart-1 27-35 in which the alanine in position 28 is replaced with leucine, and comprising a bond of the ###-homologation type between the same residues as in the case described above for the peptide analogues β1 to β9.

The invention is also the peptide analogues of MART1 27-35 in which at least one of the -CO-NH- bonds is replaced with a $-CH_2-CH_2-$ bond, such as the analogues 1 to 8 below (also referred to as carba pseudopeptide analogues of MART):

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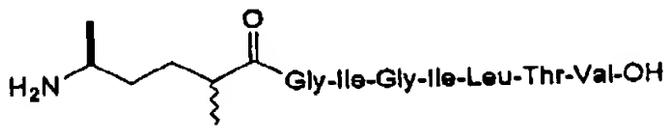

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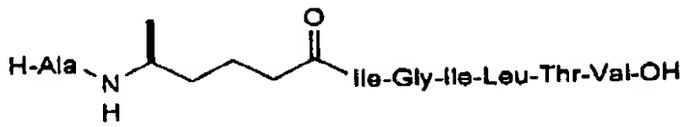

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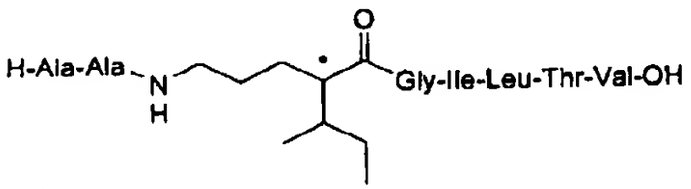

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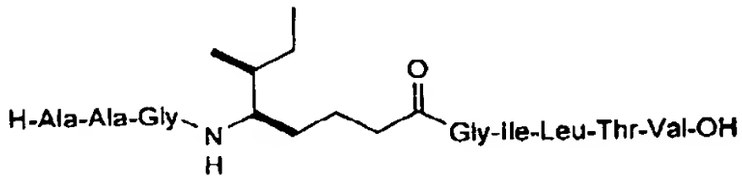
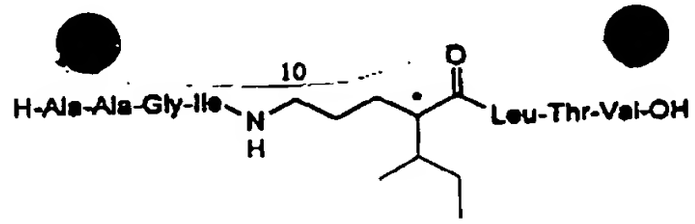
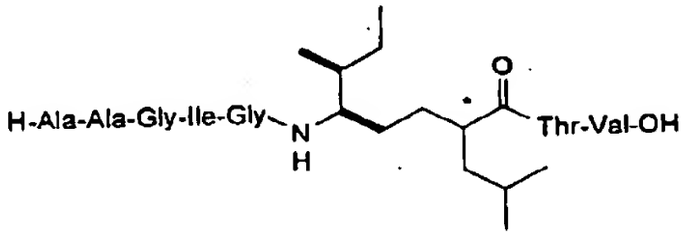
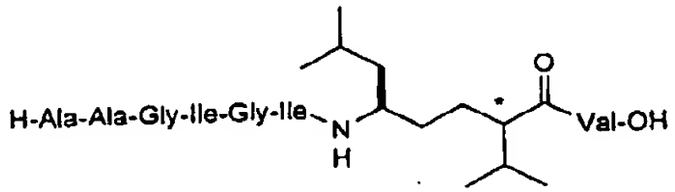
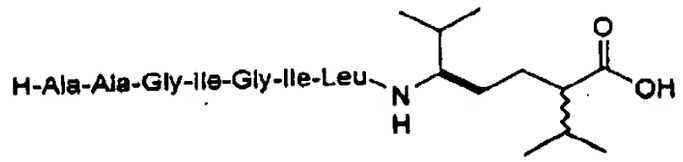










More particularly, the invention relates to the peptide analogues of the parent peptides of influenza virus, in particular of the parent peptide M58-66, the said peptide analogues corresponding to the said parent peptides in which at least one of the -CO-NH- peptide bonds is modified, with the exception of modifications of the retro or retro-inverso type ; advantageously, the said analogues are chosen from those in which at least one of the -CO-NH- bonds is replaced with a -CH_2-NH-bond, such as the following analogues :

	Séquences P1 P2 P3 P4 P5 P6 P7 P8 P9
M58-66	H- G - L - L - G - F - V - F - T - L -OH
Ψ(1-2)	H-G$\Psi_{(CH_2NH)}$L-L - G - F - V - F - T - L -OH
Ψ(2-3)	H- G- L$\Psi_{(CH_2NH)}$L- G - F - V - F - T - L -OH
Ψ(3-4)	H- G - L -L$\Psi_{(CH_2NH)}$G- F - V - F - T - L -OH
Ψ(4-5)	H- G - L - L - G$\Psi_{(CH_2NH)}$F- V - F - T - L -OH
Ψ(5-6)	H- G - L - L - G - F$\Psi_{(CH_2NH)}$V- F - T - L -OH
Ψ(6-7)	H- G - L - L - G- F - V$\Psi_{(CH_2NH)}$F- T - L -OH
Ψ(7-8)	H- G - L - L - G - F - V - F$\Psi_{(CH_2NH)}$T - L -OH
Ψ(8-9)	H- G - L - L - G - F - V - F - T$\Psi_{(CH_2NH)}$L-OH

These peptide analogues ψ (1-2), ψ (2-3), ψ (3-4), ψ (4-5), ψ (5-6), ψ (6-7), ψ (7-8) and ψ (8-9), also referred to as reduced analogues of M58-66, correspond to the latter peptide in which the -CO-NH- bond located between the residues P1 and P2, P2 and P3, P3 and P4, P4 and P5, P5 and P6, P6 and P7, P7 and P8, and P8 and P9 is replaced, in each case, with a $-CH_2-NH-$ bond.

The invention also relates to the peptide analogues of parent peptides of the AIDS virus, in particular of the peptides NEF 84-92 and GAG 77-85, the said peptide analogues corresponding to the said parent peptides in which at least one of the -CO-NH- peptide bonds is modified, with the exception of modifications of the retro or retro-inverso type, the said analogues being chosen in particular from :

- those in which at least one of the -CO-NH- bonds is replaced with a $-CH_2-NH-$, such as the analogues NEFRD1-8 of the peptide NEF, and GAGRD1-8 of the peptide GAG, below:

		Purity
NEF	AVDLSHFLK	98%
NEFRD1	AΨ(CH_2-NH)VDLSHFLK	99%
NEFRD2	AVΨ(CH_2-NH)DLSHFLK	93%
NEFRD3	AVDΨ(CH_2-NH)LSHFLK	97%
NEFRD4	AVDLΨ(CH_2-NH)SHFLK	98%
NEFRD5	AVDLSΨ(CH_2-NH)HFLK	86%
NEFRD6	AVDLSHΨ(CH_2-NH)FLK	98%
NEFRD7	AVDLSHFΨ(CH_2-NH)LK	92%
NEFRD8	AVDLSHFLΨ(CH_2-NH)K	91%

		Purity
GAG	SLYNTVATL	96%
GAGRD1	SΨ(CH_2-NH)LYNTVATL	95%
GAGRD2	SLΨ(CH_2-NH)YNTVATL	95%
GAGRD3	SLYΨ(CH_2-NH)NTVATL	95%
GAGRD4	SLYNΨ(CH_2-NH)TVATL	95%
GAGRD5	SLYNTΨ(CH_2-NH)VATL	95%
GAGRD6	SLYNTVΨ(CH_2-NH)ATL	95%
GAGRD7	SLYNTVAΨ(CH_2-NH)TL	95%
GAGRD8	SLYNTVATΨ(CH_2-NH)L	95%

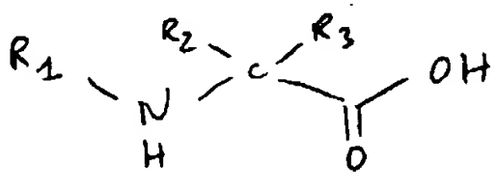
- those in which at least one of the -CO-NH- bonds is replaced with a -CHOH-NH- bond, such as the following analogues NEFHEA1-8 of the peptide NEF:

		Purity	Mass
NEF	AVDLSHFLK		
NEFHEA1	AΨ(CHOH-NH)VDLSHFLK	98%	1046.50
NEFHEA2	AVΨ(CHOH-NH)DLSHFLK	95%	1046.56
NEFHEA3	AVDΨ(CHOH-NH)LSHFLK	83%+15%	1029.00
NEFHEA4	AVDLΨ(CHOH-NH)SHFLK	98%	1047.97
NEFHEA5	AVDLSΨ(CHOH-NH)HFLK	99%	1047.08
NEFHEA6	AVDLSHΨ(CHOH-NH)FLK		
NEFHEA7	AVDLSHFΨ(CHOH-NH)LK	97%	1047.19
NEFHEA8	AVDLSHFLΨ(CHOH-NH)K	97%	1046.77

Peptide analogues which are preferred in the context of the present invention are characterized in that at least one of the amino acids in the peptide chain of the parent peptide is substituted with a non-protein-generating amino acid as defined above.

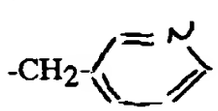
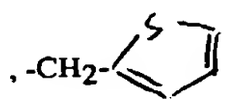
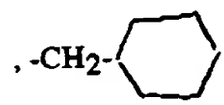
Advantageously, the non-protein-generating amino acids used are chosen from the following amino acids:

- the amino acids of D configuration,
- the α-amino acids of the general formula :



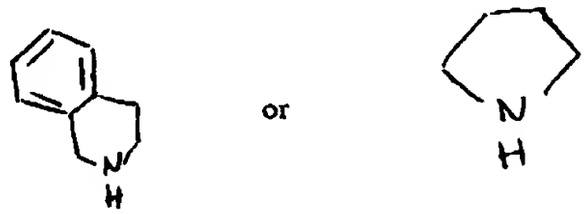
in which :

- R_1, R_2 and R_3, represent, independently of each other : a hydrogen atom, a hydroxyl, an alkyl radical of 1 to 25 carbon atoms, a radical containing an allyl group and having from 3 to 25 carbon atoms, a radical containing one or more aromatic or non-aromatic rings, in particular an aryl group, and having from 6 to 25 carbon atoms, and in particular the following groups: $-CH_3$ (methyl), $-CH_2CH_3$ (ethyl), $-(CH_2)_4-CH_3$, $-CH(CH_3)_2$ (isopropyl), $-C(CH_3)_3$ (tert-butyl), -Φ (phenyl), $-CH_2$ Φ (benzyl), $-CH_2$ Φ Cl (para-chlorobenzyl), $-CH_2-CH_2$ Φ (2-phenylethyl), $-CH_2CHCH_2$ (alkyl), methylfluorenyl, $-CH_2CONH_2$ (glycolamide), $-CH_2CON\ \Phi_2$ (benzhydrylglycolamide), -CHOHΦ,

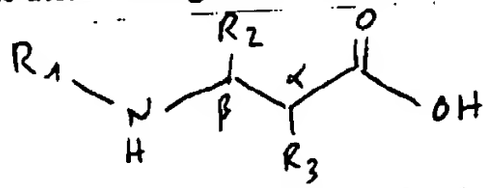




it being understood that one of the two groups R_2 and R_3 can represent a side chain of natural amino acids when either R_1 or the other of the two groups R_2 and R_3 do not represent a hydrogen atom,

- where appropriate, R_1, R_2, Cα and N form an aromatic or non-aromatic heterocycle of 4 to 8 carbon atoms, which may be substituted, in particular a heterocycle of the formula :



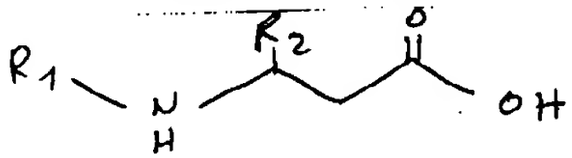
- the β-amino acids of the general formula :



in which R_1, R_2 and R_3, independently of each other, represent a side chain of a natural amino acid or are as defined above,

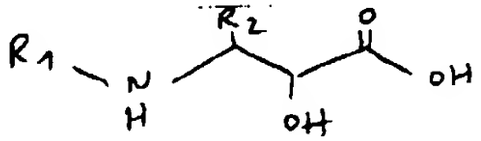
in particular :

. the β-homo amino acids of the formula:



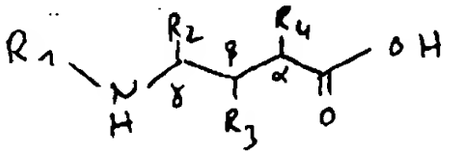
in which R_1 and R_2 are as defined above, or

. the α-hydroxy β-homo amino acids of the formula :



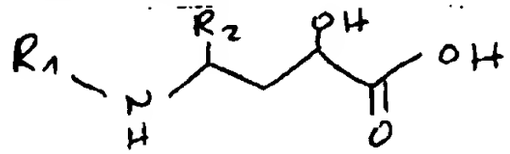
in which R_1 and R_2 are as defined above,

- the γ-amino acids of the general formula:



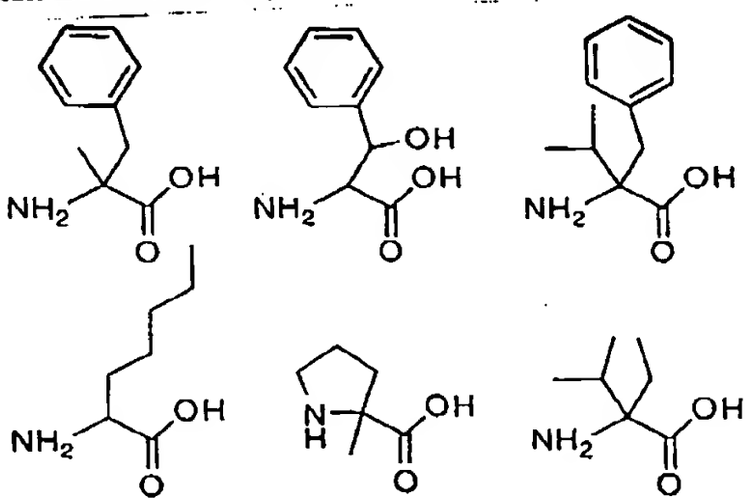
in which R_1, R_2, R_3 and R_4 represent, independently of each other, a side chain of a natural amino acid, or R_1, R_2 and R_3, are as defined above and R_4 has the same meaning as that given above for R_1, R_2 and R_3,

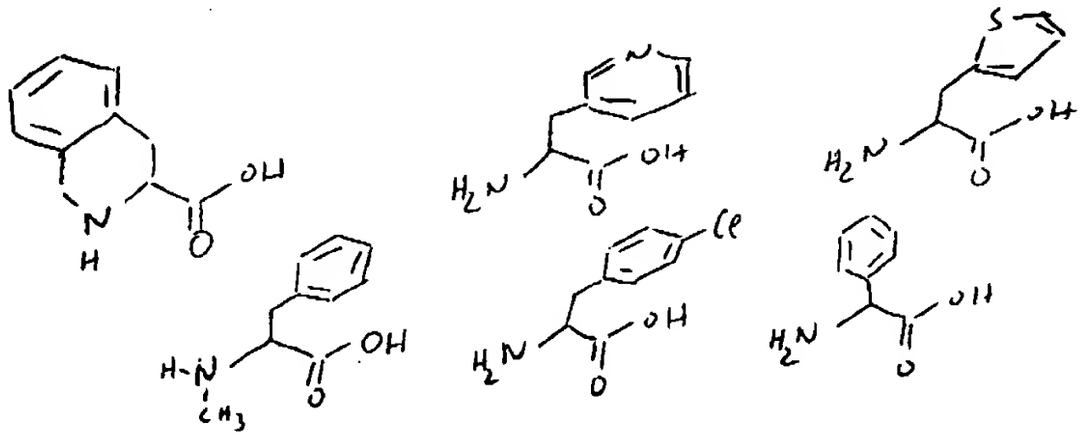
in particular the statin derivatives of the formula :



in which R_1 and R_2 are as defined above.

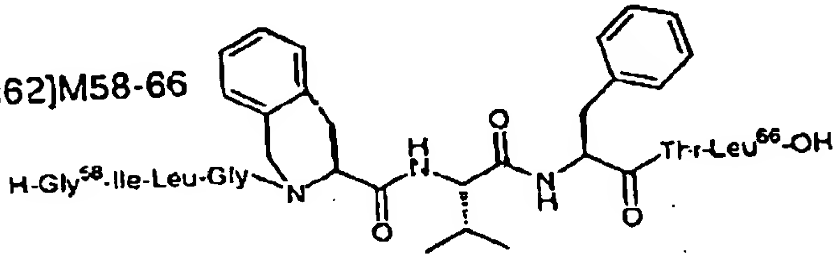
Advantageously, the non-protein-generating amino acids used in the context of the present invention are chosen from;



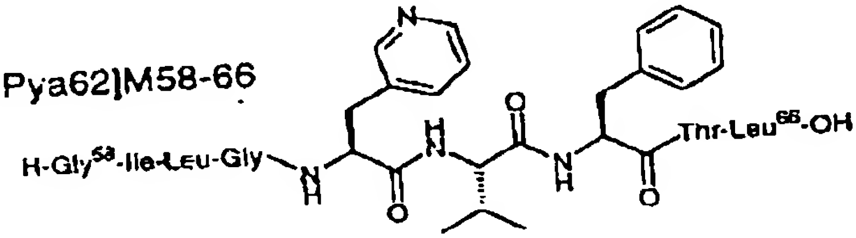


More particularly the invention relates to the peptide analogues of the parent peptides of influenza virus, in particular the parent peptide M58-66, the said peptide analogues corresponding to the said parent peptides in which at least one of the amino acids of the peptide chain is substituted with a non-protein-generating amino acid, such as the following analogues:

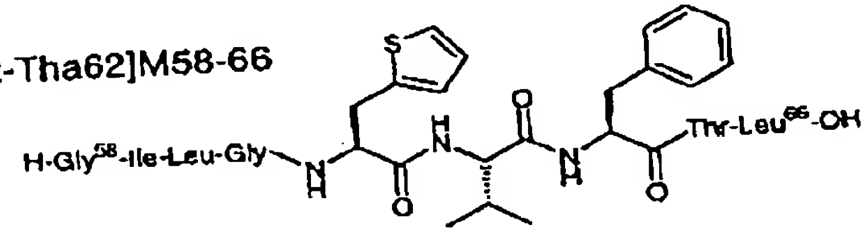
[Tic62]M58-66



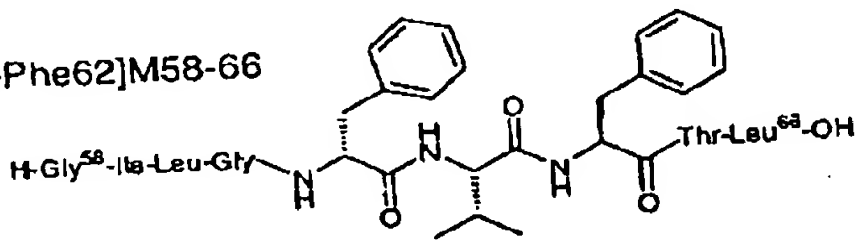
[3-Pya62]M58-66



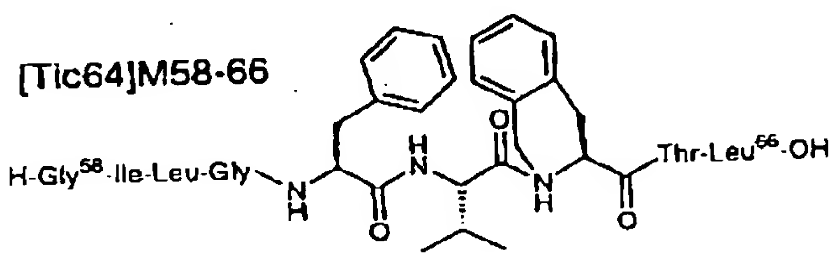
[2-Tha62]M58-66



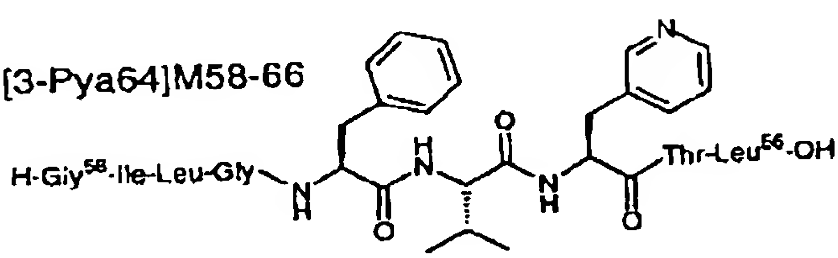
[D-Phe62]M58-66



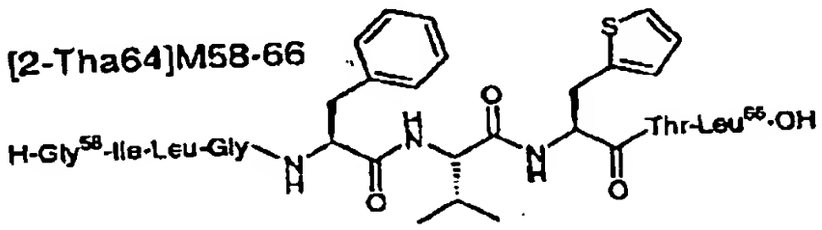
[Tic64]M58-66



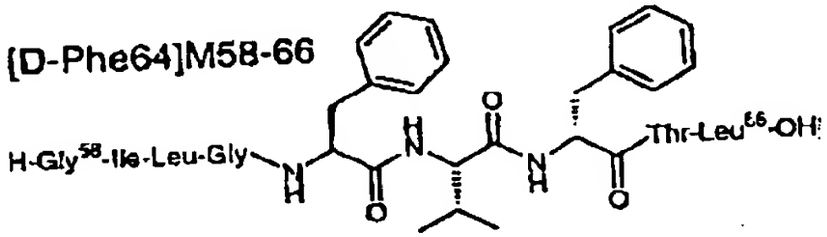
[3-Pya64]M58-66



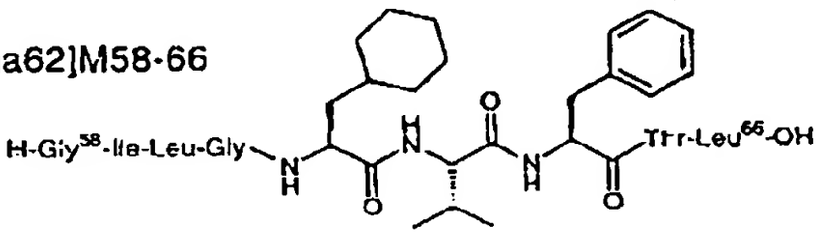
[2-Tha64]M58-66



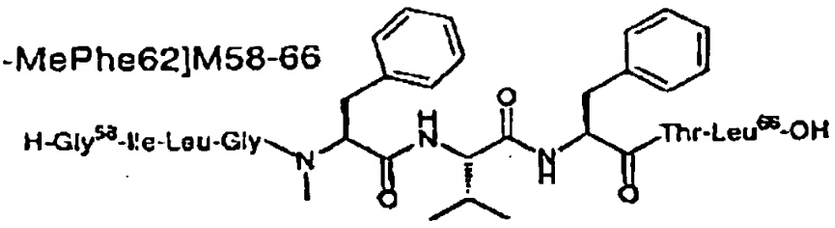
[D-Phe64]M58-66



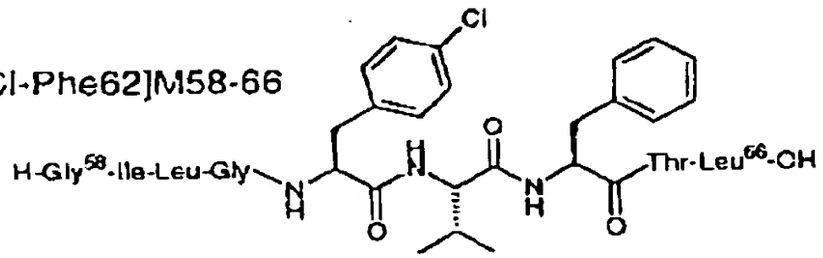
[Cha62]M58-66



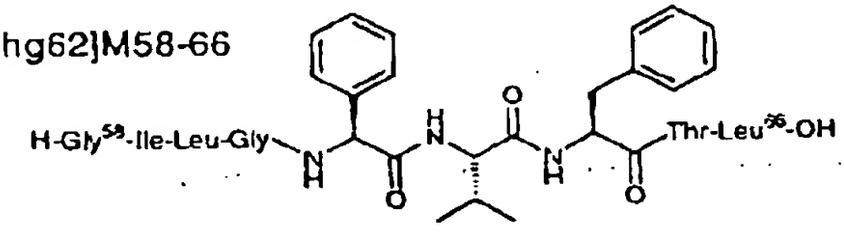
[N-MePhe62]M58-66

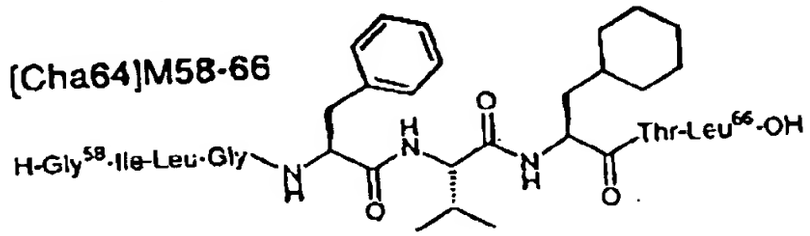


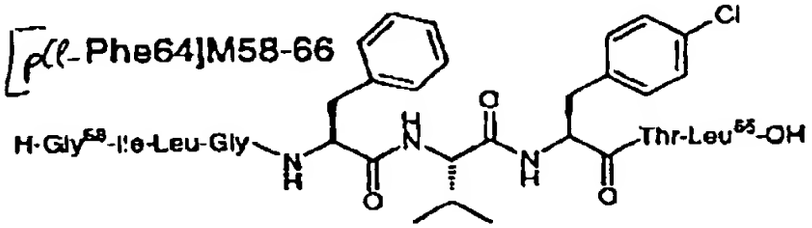
[pCl-Phe62]M58-66

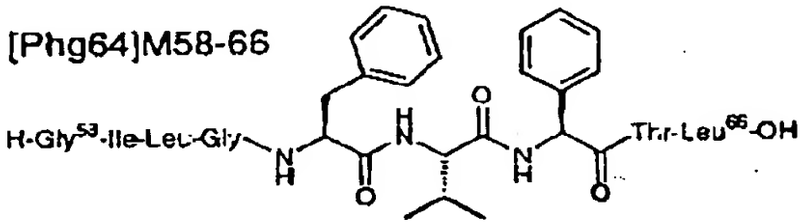


[Phg62]M58-66









Advantageously, the abovementioned peptide analogues of the invention are selected from those which are capable:

- on the one hand, of being recognized by the molecules of the MHC and of associating with these molecule, in particular by carrying out the following method :

. incubation (in particular for about 2 hours at 25°C and then for about 12 hours at 4°C) of the peptide analogue in the presence of molecules of the MHC, derived from the lysis of human or animal cells, or purified in particular by affinity chromatography from human or animal cell lines, on a solid support coated with a first antibody, in particular a monoclonal antibody, which specifically recognizes the molecules of the MHC in their conformation which is dependent on their binding to the said peptide analogue

. addition to the above solid support of a second antibody which is labelled, in particular by means of coupling with a radioactive, enzymatic or fluorescent label, the said labelled antibody specifically recognizing either the molecules of the MHC in their conformation which is dependent on their binding to the peptide analogue, or a molecule which itself binds specifically to the molecules of the MHC in their abovementioned conformation, in particular β2-microglobulin which specifically recognizes the molecules of the MHC of category I,

. detection, after rinsing the solid support, of the possible presence of the second labelled antibody which has remained bound to the solid support, thereby demonstrating an effect of recognition and association between the molecules of the MHC and the peptide analogue studied,

- and, on the other hand, of forming a complex with the said molecules of the MHC, the stability of which complex can be evaluated by carrying out a method for monitoring over time the binding established between the peptide analogue and the molecules of the MHC, this method advantageously being carrying out according to a protocol which is identical to the above method, but in which the step of incubation of the peptide analogue in the presence of the molecules of the MHC on the solid support coated with the said first antibody is carried out (advantageously at a temperature of 37°C) for times ranging from a few minutes to several days.

The peptide analogues of the invention must be recognized by the molecules of the MHC and must associate with these molecules, in particular in the context of carrying out the recognition test described above. This association can be weak (detectable at peptide analogue concentrations of the order of 10^{-4} to 10^{-5} M), intermediate (detectable at peptide analogue concentrations of the order of 10^{-5} to 10^{-7} M), or strong (detectable at peptide analogue concentrations of the order of 10^{-8} to 10^{-9} M).

The peptide analogues recognized by the molecules of MHC in the context of the present invention are preferably capable of binding for at least about 30 minutes to the said molecules of the MHC.

More particularly, the invention relates to the peptide analogues as described above and characterized in that they are selected from those which are capable :

- of inducing *in vitro* the appearance and growth of cytotoxic T lymphocytes from human or animal cells, in particular from peripheral blood mononuclear cells (PBMCs) in the presence of factors required for the growth and differentation of the cytotoxic T cells,
- of inducing *in vitro* cytolysis, by means of cytotoxic T lymphocytes, of target cells carrying at their surface the peptide analogue associated with the molecules of the MHC, the said cytotoxic T lymphocytes advantageously being taken from a patient suffering from a pathology in which the parent peptide of the peptide analogue studied is involved,
- and of inducing *in vitro* the secretion of cytokines (or interleukins) by means of the abovementioned cytotoxic T lymphocytes, in particular IL-2, IL-4 or γ-interferon,

the said peptide analogues thus selected being :

. either receptor agonists (TCR) which recognize the antigen (i.e. the parent peptide) of the cytotoxic T cells, and are derived from parent peptides which themselves behave as agonists or antagonists of the said receptors,

. or partial agonists of the said receptors, and are derived from parent peptides which themselves behave as agonists of the said receptors, these partial agonists inducing, in particular, the secretion of one or more cytokines other than those whose secretion is induced by means of the parent peptides.

The invention relates more particularly to the peptide analogues as described above and characterized in that they are selected from those :

- which are capable of inducing *in vitro* the appearance and growth of cytotoxic T lymphocytes from human or animal cells, in particular from peripheral blood mononuclear cells (PBMCs), in the presence of factors required for the growth and differentiation of cytotoxic T cells,
- which do not induce *in vitro* the cytolysis, by means of cytotoxic T lymphocytes, of target cells carrying at their surface the peptide analogue associated with the molecules of the MHC, the said cytotoxic T lymphocytes advantageously being taken from a patient suffering from a pathology in which the parent peptide of the peptide analogue studied is involved,
- which do not induce *in vitro* the secretion of cytokines (or interleukins) by means of the abovementioned cytotoxic T lymphocytes, in particular IL-2, IL-4 or γ-interferon,

the said peptide analogues thus selected being antagonists of the cytotoxic T cell receptors.

The invention also relates to the use of peptide analogues as defined above for the preparation of medicinal products, in particular vaccines, intended for preventing or treating pathologies in which the parent peptides are agonists or antagonists of receptors which recognize the antigen of the cytotoxic T cells, and more particularly neurodegenerative pathologies which are infectious (of viral or bacterial origin), tumoural, autoimmune and allergic.

Among the tumoural pathologies which can be treated in the context of the present invention, mention may be made of melanoma, in particular with the aid of the peptide analogues of the peptide MART1 27-35 which are described above.

Among the diseases of viral origin which can be prevented or treated in the context of the present invention, mention may be made of :

- AIDS brought about by the human immunodeficiency virus HIV1 or HIV2, in particular with the aid of peptide analogues of the peptides NEF and GAG which are described above,
- paraplegia associated with HTVL-1, or T-cell leukemia in adults, brought about by the human T-cell leukemia virus (HTLV virus),

- infections brought about by the syncitial respiratory virus,
- infections brought about by coxsakie virus, for example acute lymphocytic meningitis,
- infections brought about by the Epstein-Barr virus, for example infectious mononucleosis,
- infections brought about by cytomegalovirus, for example cytomegalic inclusion disease,
- herpes brought about by the human herpes virus,
- herpes brought about by virus 6 of herpes simplex,
- infections brought about by human B19 parvovirus, for example infectious gastro-enteritis,
- hepatitis B brought about by the hepatitis B virus,
- hepatitis C brought about by the hepatitis C virus,
- influenza brought about by the influenza virus, in particular with the aid of peptide analogues of the peptide M58-66 which are described above,
- German measles brought about by the rubella virus,
- infections brought about by the Dengue virus, for example arboviruses,
- colds, rhinitis, and coryza brought about by rhinoviruses,
- aphtous fever brought about by the aphthous fever virus,
- cervical cancer brought about by the pillomavirus (HPV).

Among the main autoimmune diseases which can be treated in the context of the present invention, mention may be made of those collated in Table A below.

Table A

Main autoimmune diseases (from top to bottom, autoimmune diseases of specific organs to autoimmune diseases not of specific organs).

Disease	Autoantigen involved
Hashimoto's thyroiditis	Thyroglobulin, microsomes
Basedow's disease	TSH receptor
Addison's disease	Corticoadrenal
Hypophyseal insufficiency	Pituitary
Biermer's gastritis	Parietal cell of the stomach
	Intrinsic factor
Certain sterilities	Spermatozoa, ovaries
Type 1 juvenile diabetes	Islets of Langerhans, insulin
Goodpasture's syndrome	Glomerular basal membrane
Myasthenia	Striated muscle, acetylcholine receptor
Acute articular rheumatism	Myocardium (streptococci)
Pemphigus	Epidermal intercellular bridges
Bullous pemphigoid	Cutaneous basal membrane
Herpetiform dermatitis	Gliadin, reticulin
Vitiligo	Melanocytes
Pelade	Hair follicle
Psoriasis	
Sympathic ophthalmia	Uvea
Uveitis	Anterior chamber of the eye
Guillain-Barré's syndrome	
Multiple sclerosis	Myelin
Haemolytic anaemia	Red blood cells
Idiopathic thrombopaenic Purpura	Platelets
Idiopathic leukopaenia	Granulocytes
Primitive biliary cirrhosis	Mitochondria
Active chronic hepatitis	Smooth muscle, nuclei
Haemorrhagic rectocolitis	
Crohn's ileitis	Colon (*E. coli*)
Gougerot-Sjögren's syndrome	Nuclei: SS-A, SS-B

Rheumatoid arthritis	IgG, nuclei
Dermatopolymyositis	Nuclei: Jo1, muscles
Scleroderma	Nuclei: Scl-70
Mixed connectivity	Nuclei: RNP
Discoid lupus erythematosus	Nuclei:
Disseminated lupus erythematosus	Nuclei: DNA, Sm antigen
	Clotting factors
	Cardiolipin, etc.

The invention also relates to any pharmaceutical composition, characterized in that it comprises, as active principle, at least one peptide analogue (agonist, which may be a partial agonist, or antagonist) as defined above, in combination with a pharmaceutically acceptable vehicle.

Advantageously, the pharmaceutical compositions according to the invention are in a form which can be administered orally or parenterally, in particular at a rate of about 500 µg to 5 mg at a time, in particular at a rate of 3 times a day.

More particularly, the invention relates to the pharmaceutical compositions as described above, containing, as active principle, at least one antagonist according to the invention, and their use in the context of treating autoimmune diseases.

More particularly, the invention furthermore relates to the pharmaceutical compositions as described above, containing, as active principle, at least one partial agonist according to the invention, and their use in the context of treating allergic diseases.

The invention also relates to any vaccine, characterized in that it comprises, as active principle, at least one peptide analogue, preferably an agonist peptide analogue, which may be a partial agonist, as defined above, in combination with a pharmaceutically acceptable vehicle.

Advantageously, the vaccines according to the invention are in a form which can be administered orally or parenterally, in particular at a rate of about 500 µg to 5 mg at a time, in particular at a rate of 3 times a day.

The invention also relates to any composition intended for *in vivo* diagnosis of abovementioned pathologies involving the immune response to cell mediation, in particular the cytotoxic T lymphocytes, or to the *in vivo* evaluation of the immune response in the context of the said pathologies, by carrying out a skin hypersensitivity reaction by means of intradermal injection of the said diagnostic composition, characterized in that it comprises at least one peptide analogue, preferably an agonist peptide analogue, which may be a partial agonist, as defined above, in combination with a biologically acceptable vehicle.

The invention also relates to the use of peptide analogues as described above for the preparation of the said compositions intended for the *in vivo* diagnosis of the abovementioned pathologies.

The invention also relates to complexes between a peptide analogue as defined above and a molecule of the major histocompatibility complex (also referred to as MHC-peptide analogue binary complexes).

The immune response involves the recognition of an endogenous or exogenous antigen by specialized cells. In order to be recognized, the antigen must first be presented in a suitable manner by antigen presenting cells (APCs). Whereas the B lymphocytes recognize epitopes borne by the unmodified intact antigens, the presentation of the antigen to the T lymphocytes is more complex since the antigen is first internalized by the presenting cell, proteolysed and then possibly re-expressed at its surface in the form of peptide fragments in association with the proteins of the major histocompatibility complex (MHC). The T lymphocyte, which does not recognize the native antigen, recognizes a peptide fragment associated with an MHC molecule.

These MHC molecules belong essentially to two categories: I and II.

The molecules of category I are transmembrane glycoproteins consisting of a heavy polymorphic α chain associated non-covalently with a light non-glycosylated β2m chain. Their crystallographic structure has been resolved (Bjorkman et al. (1987), Nature, 329: 506-512), and shows the presence of a channel forming the presentation site of the peptide, the base of which is composed of eight β sheets and the edges are composed of two α helices. These molecules are presented at the surface of virtually all cells.

The molecules of category II are also membrane glycoproteins consisting of two polymorphic chains α et β which are non-covalently linked to form, as shown in the crystallographic structure recently elucidated (Brown et al. (1993), Nature, 364: 33-39), a β-pleated platform supporting two α helices. The channel formed is the presentation site of the peptide. These molecules are expressed only at the surface of certain cells, including macrophages and B cells.

The cytotoxic T lymphocytes (cells possessing CD8 labels) recognize proteolytic fragments of viral proteins associated with the MHC molecules of category I and bring about the lysis of cells bearing the antigen.

The helper T lymphocytes (cells bearing CD4 labels) recognize fragments of exogenous proteins captured by endocytosis which are presented in association with the MHC molecules of category II and induce the cellular stimulation of the immune response.

The invention also relates to complexes between a peptide analogue according to the invention and a T cell receptor (which are also referred to as T receptor-peptide analogue complexes).

The invention is also directed towards complexes between a molecule of the major histocompatibility complex, a peptide analogue as defined above and a T cell receptor (which is also referred to as an MHC-peptide analogue-T receptor ternary complex).

The invention also relates to the use of peptide analogues as defined above, for carrying out a method for the *in vitro* diagnosis of the pathologies mentioned above.

More particularly, the invention relates to any method for the *in vitro* diagnosis of pathologies involving the immune response to cell mediation, in particular the cytotoxic T lymphocytes, i.e. pathologies associated with the presence, in a patient's body, of exogenous or endogenous peptides which can interact with molecules of the MHC, and which are liable to be directly or indirectly involved in the process of development of these pathologies in man or animals, characterized in that it comprises:

- placing a biological sample obtained from a patient, in particular the blood or any biological sample liable to contain lymphocytes, in contact with a peptide analogue as defined above, under conditions which allow reaction between the receptors of the T cells liable to be present in the biological sample, and the abovementioned binary complex formed between the said peptide analogue and the molecules of the MHC present in the said sample;
- the *in vitro* detection of the MHC-peptide analogue-T receptor ternary complex which may be formed in the preceding step.

The abovementioned diagnostic methods of the invention are advantageously carried out in the following way:

- incubation of the said biological sample with peptide analogues according to the invention, the said peptide analogues being bound to a solid support, in particular to the inside of the wells of microtitration plates of the type usually used for carrying out detection or assay techniques which are well known under the name ELISA (Enzyme Linked Immuno Sorbent Assay),
- rinsing of the solid support,
- incubation of the components which have remained bound to the solid support after the preceding rinsing step, with a medium containing antibodies, in particular antibodies raised against the ternary complex according to the invention, which are labelled (in particular by radioactive, enzymatic or fluorescent means) or which are capable of being recognized in turn by a labelled reagent,
- rinsing of the solid support,

- detection of the labelled antibodies which have respectively remained bound to the ternary complexes during the preceding incubation step.

A subject of the invention is also equipment or kits for carrying out the methods of *in vitro* diagnosis as described above, comprising:

- a peptide analogue as defined above;
- reagents for making a medium suitable for forming an immunological reaction;
- reagents for detecting the ternary complex according to the invention, which has been produced after the immunological reaction, the said reagents optionally containing a label or being capable of being recognized in turn by a labelled reagent, more particularly in the case in which the peptide analogue is not labelled.

The invention also relates to antibodies directed against the MHC-peptide analogue binary complexes as defined above, the said antibodies being as obtained by immunizing an animal with at least one of the abovementioned complexes, the said antibodies being capable of forming a complex with these binary complexes.

The antibodies according to the invention are monoclonal or polyclonal antibodies.

The abovementioned polyclonal antibodies are obtained by immunizing an animal with at least one MHC-peptide analogue complex according to the invention, followed by recovery of the desired antibodies in purified form, by taking the serum of the said animal and separating out the antibodies from the other constituents of the serum, in particular by affinity chromatography on a column onto which is bound an antigen which is specifically recognized by the antibodies, in particular an MHC-peptide analogue complex according to the invention.

The monoclonal antibodies according to the invention can be obtained by the hybridoma technique, the general principle of which is recalled below.

In a first stage, an animal, generally a mouse (or cells under culture in the context of *in vitro* immunizations), is immunized with an MHC-peptide analogue complex according to the invention, the B lymphocytes of which are then capable of producing antibodies against the said complex. These antibody-producing lymphocytes are then fused with (in particular mouse) "immortal" myeloma cells to give rise to hybridomas. Using the heterogeneous mixture of cells thus obtained, a selection is then made of cells capable of producing a particular antibody and of multiplying indefinitely. Each hybridoma is multiplied in the form of a clone, each leading to the production of a monoclonal antibody whose properties of recognition with respect to the MHC-peptide analogue complex of the invention may be tested by ELISA, for example, by one- or two-dimensional immunotransfer, by immunofluorescence, or with the aid of a biosensor. The monoclonal antibodies thus

selected are then purified, in particular according to the affinity chromatography technique described above.

The antibodies according to the invention are more particularly characterized in that they are capable of forming a complex with MHC-peptide analogue complexes and/or with complexes of MHC-parent peptide or protein correspondant to the said peptide analogues.

Advantageously, the antibodies raised against the MHC-peptide analogue complexes of the invention recognize the abovementioned complexes of MHC-parent peptide or protein, with an affinity which is at least equal to that presented by the anti(MHC-parent peptide or protein complex) antibodies with respect to the MHC-parent peptide or protein complexes.

The affinity which is concerned above can be measured by means of the affinity constant at equilibrium, Ka, of the complexes involving one of the abovementioned antibodies with one of the abovementioned complexes.

The invention also relates to any pharmaceutical composition, characterized in that it comprises antibodies as defined above, in combination with a physiologically acceptable vehicle, as well as their use in the context of treating the abovementioned pathologies.

The invention also relates to the process for screening peptide analogues as defined above, characterized in that it comprises the following steps :

. incubation (in particular at a temperature of 37°C), for times ranging from a few minutes to several days, of the peptide analogue in the presence of molecules of the MHC, derived from the lysis of human or animal cells, or purified in particular by affinity chromatography from human or animal cell lines, on a solid support coated with a first antibody, in particular a monoclonal antibody, which specifically recognizes the molecules of the MHC in their conformation which is dependent on their binding to the said peptide,

. addition to the preceding solid support of a second antibody which is labelled, in particular by means of coupling with a radioactive, enzymatic or fluorescent label, the said labelled antibody specifically recognizing either the molecules of the MHC in their conformation which is dependent on their binding to the peptide analogue, or a molecule which itself binds specifically to the molecules of the MHC in their abovementioned conformation, in particular β2-microglobulin which specifically recognizes the molecules of the MHC of category I,

. detection, after rinsing the solid support, of the possible presence of the second labelled antibody which has remained bound to the solid support,

. evaluation of the duration of the association between the said peptide analogue and the molecules of the MHC.

The invention also relates to any set or kit for carrying out a process for screening peptide analogues as defined above, comprising:

- molecules of the MHC, and/or
- antibodies which specifically recognize the molecules of the MHC in their conformation which is dependent on their binding to the said peptide analogue, which antibodies are advantageously bound to a solid support, or are supplied with the reagents required for binding them to the solid support, and/or
- antibodies which are labelled, in particular by means of coupling with a radioactive, enzymatic or fluorescent label, these antibodies specifically recognizing either the molecules of the MHC in their conformation which is dependent on their binding to the peptide analogue, or a molecule which itself binds specifically to the molecules of the MHC in their abovementioned conformation, in particular β2-microglobulin which specifically recognizes the molecules of the MHC of category I, and/or
- a protocol for carrying out the said process, and/or
- a control peptide.

The invention will be further illustrated with the aid of the following examples of the production and study of the peptide analogues as described above.

I- Synthesis of the peptide analogues of the invention :

A) General features :

By way of illustration, the liquid-phase synthesis method consists in successively condensing the aminoacyls two by two in the required order, or in condensing aminoacyls and fragments which have been formed beforehand and which already contain several aminoacyls in the appropriate order, or alternatively several fragments thus prepared beforehand, it being understood that care will have been taken to protect beforehand all the reactive functions borne by these aminoacyls or fragments, with the exception of the amine functions of one and carboxyl of the other or vice-versa, which should normally be involved in the formation of the peptide bonds, in particular after activation of the carboxyl function, according to the methods which are well known in peptide synthesis.

It will be possible, for example, to use protecting groups of urethane type (Boc, Fmoc benzyloxycarbonyl or allyloxycarbonyl) to protect the N-terminal ends of the amino acids, and groups of ester type (methyl, ethyl, benzyl, tert-butyl, allyl or alternatively benzhydrylglycolamide) to protect the C-terminal ends of the amino acids.

Such a synthesis can be carried out by first condensing the aminoacyl residue AA1, whose COOH fonction is protected, with the aminoacyl residue AA2 whose NH_2 function is protected. The amine function of the residue AA2 in the fragment AA2-AA1 thus obtained is then deprotected, in order subsequently to condense the said fragment with the aminoacyl residue AA3 whose amine function is protected. The preceding steps are repeated as many times as there are aminoacyl residues to be introduced into the chain of the retro analogues to be synthesized.

According to another preferred method of the invention, use is made of the technique described by R.D. Merrifieldin the article entitled "Solid phase peptide synthesis" (J. Am. Chem. Soc. (1963), **85**, 2149-2154).

In order to manufacture a peptide chain according to the Merrifield process, use is made of a highly porous polymer resin, onto which is bound the first C-terminal amino acid (in this instance AA1-OH) of the chain. This amino acid is bound to the resin via its carboxyl group and its amine function is protected, for example with the t-butoxycarbonyl group.

When the first C-terminal amino acid is thus bound to the resin, the protecting group is removed from the amine function by washing the resin with an acid.

The amino acids which will make up the peptide chain are thus bound, one after the other, to the amino group each time the portion of the peptide chain which is already formed, and which is attached to the resin, undergoes preliminary deprotection.

When all of the desired peptide chain is formed, the protecting groups are removed from the various amino acids constituting the peptide chain and the peptide is detached from the resin, for example using hydrofluoric acid.

B) Particular case of the peptide analogues of the invention :

One or more of the synthesis steps described above can be interrupted in order to insert a bond other than the -CO-NH- bond between certain aminoacyl residues, and /or one or more non-protein-generating amino acids.

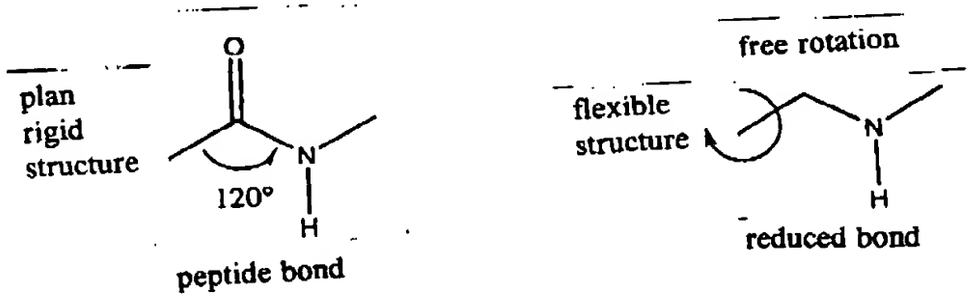
B.1.) Synthesis of peptide analogues containing modified peptide bonds :

The notation ψ (Spatola & Darlak, 1988) is generally used to denote the pseudopeptide bond which is replacing the amide bond (CONH). Among the modifications most frequently encountered, mention may be made, for example, of the following, the syntheses of which are detailed in the bibliographic references given : the methyleneamino (reduced) bond ψ[CH_2NH] (Szelke *et al.*, 1982 ; Martinez *et al.*, 1985 ; Sasaki & Coy, 1987) ; retro-inverso bond ψ[NHCO] (Shemyakin *et al.*, 1969 ; Goodman & Chorev, 1979 ; Chorev & Goodman, 1993);

olefin bond ψ[CH=CH] (Hann *et al.*, 1982 ; Kempf *et al.*, 1991 ; Bohnstedt *et al.*, 1993) ; carba bond ψ[CH_2CH_2] (Rodriguez *et al.*, 1990a, 1990b) ; methyleneoxy (ether) bond ψ[CH_2O] (TenBrink, 1986 ; Breton *et al.*, 1990) ; ketomethylene bond ψ[$COCH_2$] (Harbeson & Rich, 1989 ; Gonzalez-Muniz *et al.*, 1995), hydroxyethylene bond ψ[$CH(OH)CH_2$] (Evans *et al.*, 1985 ; Wuts *et al.*, 1992).

1) Synthesis of the reduced pseudopeptides corresponding to the sequences of MART and of the matrix peptide M58-66 :

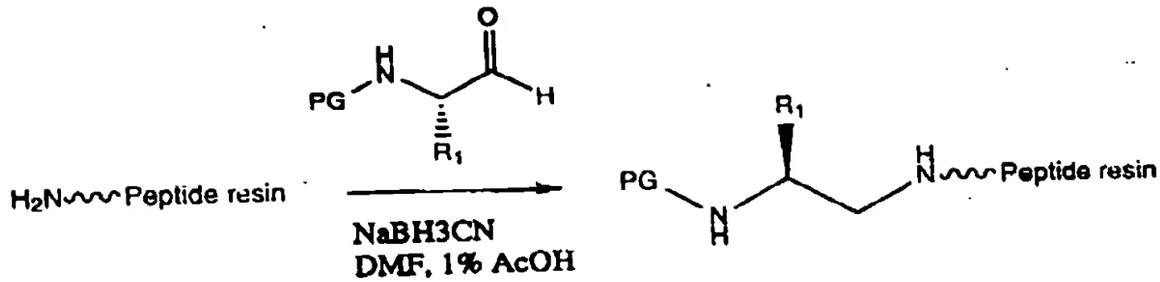
The contribution of the amine function of each peptide bond of the antigen Mart-1 27-35 (AAGIGILTV) of the protein Mart-1 du melanoma and of the antigen M58-66 (GLLGFVFTL) of the matrix protein of influenza virus was evaluated as regards the interactions of these antigens with their receptors: the molecule of category I of the MHC HLA-A2 and TCR.



Scheme 1: Schematic representation of the peptide bonds and reduced bonds.

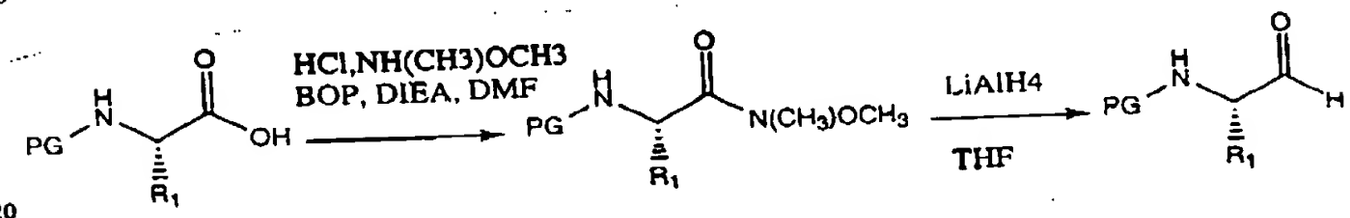
The reduced bond, or methyleneamino bond, has the particular feature of being more flexible than the peptide bond on account of a free rotation of the carbon-carbon bond (scheme 1). The introduction of a reduced bond can locally modify the orientation of the amino acid side chains. The reduced bond can exist in its protonated form at physiological pH. The changes brought about by the presence of a reduced bond, i.e. the change in the structure and the change in the hydrophilic nature of the parent peptide, can have implications in the phenomena of binding and recognition of the antigen.

The synthesis of each reduced peptide chain was performed in solid phase and by standard Fmoc chemistry. The reduced bond is obtained by condensing an N-protected α-amino aldehyde with the α-amino acid immobilized on the resin. The imine form is reduced *in situ* with sodium cyanoborohydride to give the reduced bond according to the method described by Sasaki and Coy in 1987 (scheme 2).



Scheme 2: Incorporation of the reduced bond in solid phase. PG: Fmoc protecting group.

The α-amino aldehyde is synthesized by the method of Fehrentz and Castro, 1983. This method allows the rapid production, without racemization, of the N-protected α-amino aldehyde from the N,O-dimethylhydroxamate of the corresponding N-protected amino acid (scheme 3).



Scheme 3: Synthesis of N-protected α-amino aldehydes by the method of Fehrentz and Castro. PG: Fmoc protecting group.

The following amino acids of the sequence of the reduced peptide are coupled in a conventional manner. After synthesis, the reduced peptide is deprotected and detached from the resin with trifluoroacetic acid (TFA). Each reduced peptide is purified by reverse-phase high pressure liquid chromatography (RP-HPLC). The mass of each peptide is controlled by matrix assisted laser desorption (MALDI) with the aid of a Bruker Protein TOF spectrometer.

The retention times (Tr) on an RP-HPLC C18 column in a gradient of from 5 to 65 % B over 30 min (with A= water containing 0.1% TFA and B= acetonitrile (ACN) containing 0.08% TFA) and the masses of the various peptides are given in Tables 1 et 2.

Table 1: Sequences and characteristics of the reduced peptide analogues of Mart-1 27-35

	Sequences P1 P2 P3 P4 P5 P6 P7 P8 P9	Tr	mass
MART1 27-35	H-A-A-G-I-G-I-L-T-V-OH	12.74	814.4
Ψ(1-2)	H-AΨ(CH_2NH)A-G-I-G-I-L-T-V-OH	12.53	802.7
Ψ(2-3)	H-A-AΨ(CH_2NH)G-I-G-I-L-T-V-OH	12.58	802.9
Ψ(3-4)	H-A-A-GΨ(CH_2NH)I-G-I-L-T-V-OH	11.85	801.6
Ψ(4-5)	H-A-A-G-IΨ(CH_2NH)G-I-L-T-V-OH	12.03	801.5
Ψ(5-6)	H-A-A-G-I-GΨ(CH_2NH)I-L-T-V-OH	11.29	801.4
Ψ(6-7)	H-A-A-G-I-G-IΨ(CH_2NH)L-T-V-OH	12.00	800.4
Ψ(7-8)	H-A-A-G-I-G-I-LΨ(CH_2NH)T-V-OH	12.77	801.5
Ψ(8-9)	H-A-A-G-I-G-I-L-TΨ(CH_2NH)V-OH	12.46	801.3

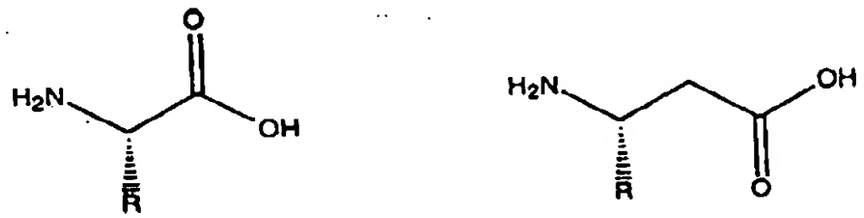
Table 2: Sequences and characteristics of the reduced peptide analogues of M58-66

	Sequences P1 P2 P3 P4 P5 P6 P7 P8 P9	Tr	mass
M58-66	H-G-L-L-G-F-V-F-T-L-OH	12.35*	968.0
Ψ(1-2)	H-GΨ(CH_2NH)L-L-G-F-V-F-T-L-OH	10.67*	956.2
Ψ(2-3)	H-G-LΨ(CH_2NH)L-G-F-V-F-T-L-OH	11.18*	957.1
Ψ(3-4)	H-G-L-LΨ(CH_2NH)G-F-V-F-T-L-OH	16.01	957.3
Ψ(4-5)	H-G-L-L-GΨ(CH_2NH)F-V-F-T-L-OH	15.61	954.3
Ψ(5-6)	H-G-L-L-G-FΨ(CH_2NH)V-F-T-L-OH	10.80*	955.8
Ψ(6-7)	H-G-L-L-G-F-VΨ(CH_2NH)F-T-L-OH	11.85*	953.2
Ψ(7-8)	H-G-L-L-G-F-V-FΨ(CH_2NH)T-L-OH	13.05*	954.7
Ψ(8-9)	H-G-L-L-G-F-V-F-TΨ(CH_2NH)L-OH	11.21*	957.3

Retention time in the gradient of from 20 to 80% B over 30 min (with A= water containing 0.1% TFA and B= ACN containing 0.08% TFA).

2) Synthesis of the β analogues of the parent MART peptide and of the mutated (Leu^{28}) MART peptide :

The β analogues are obtained by coupling β-homo amino acids in place of the natural amino acids.



aminoacid β-homo amino acid

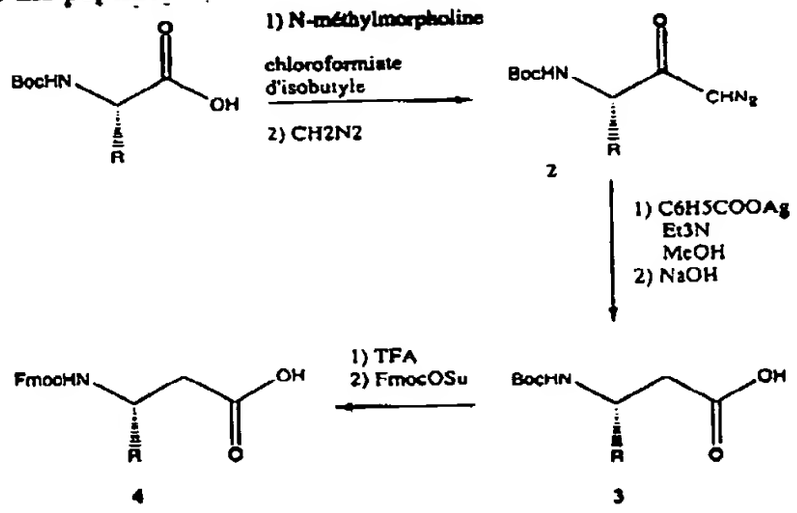
Scheme 4: Schematic representation of an amino acid and of a β-homo amino acid

The β-homo amino acid is an amino acid in which a methylene group has been inserted between the α carbon and the carboxyl. The configuration of the Cα is unchanged (scheme 4). The incorporation of a β-homo amino acid in place of an amino acid in a peptide sequence has the consequence of lengthening the peptide chain of the resulting peptide analogues. These longer peptide analogues can be made to twist and brace themselves to penetrate into the binding pocket of the molecule of category I of the MHC. In this case, certain side chains can be displaced laterally or can have their orientation modified, which can result in preferential interactions with the molecule of category I of the MHC and/or TCR.

The β-homo amino acids are obtained by chemical synthesis in several steps starting with the corresponding N-protected amino acids, according to the scheme below (scheme 5).

The amino acid protected on its amine function with a Boc group is, in a first stage, activated by means of the mixed anhydride method. The activated amino acid reacts with diazomethane to give the diazomethyl ketone 2. The Wolff rearrangement of this diazomethyl ketone in methanol, in the presence of silver benzoate and triethylamine, gives the β-homo methyl ester of the amino acid protected with the Boc group. A saponification gives the compound 3. Deprotection of the Boc, followed by reprotection of the amine function with the Fmoc are the final steps for obtaining the β-homo amino acid protected with Fmoc.

Compound 4 is introduced by means of the conventional methods for coupling amino acids in the course of solid-phase synthesis in the Fmoc strategy. In practice, 5 equivalents of the mixture (4/BOP/HOBt) are coupled for 20 min in DMF. The Fmoc group is deprotected with 50% piperidine in DMF and the synthesis is continued conventionally. See the preceding paragraph for the detachment and deprotection of the peptide, as well as for the purification and characterization of the peptides.



Scheme 5: Scheme of synthesis of Fmoc-β-homo amino acids

Tables 3 and 4 give the retention times on RP-HPLC of the ### peptides on a C18 column (gradient of from 5 to 65% B over 30 min with A= water containing 0.1% TFA and B= ACN containing 0.08% TFA) as well as their mass analysis by MALDI.

Table 3: Sequences and characteristics of the β peptide analogues of Mart-1 27-35

	Sequences P1 P2 P3 P4 P5 P6 P7 P8 P9	Rt	mass
MART1 27-35	H- A - A - G - I - G - I - L - T - V -OH	12.74	814.4
β1	H-β-homoA-A- G - I - G - I - L - T - V -OH	12.62	868.9*
β2	H- A-β-homoA- G - I - G - I - L - T - V -OH	12.50	829.9
β3	H- A - A -β-homoG-I - G - I - L - T - V -OH	12.48	828.9
β4	H- A - A - G -β-homoI-G - I - L - T - V -OH	12.50	829.5
β5	H- A - A - G - I -β-homoG-I - L - T - V -OH	12.50	828.9
β6	H- A - A - G - I - G -β-homoI-L - T - V -OH	12.64	829.0
β7	H- A - A - G - I - G - I -β-homoL-T - V -OH	12.10	828.9
β8	H- A - A - G - I - G - I - L -β-homoT-V -OH	11.92	829.1
β9	H- A - A - G - I - G - I - L - T -β-homoV-OH	12.68	829.2

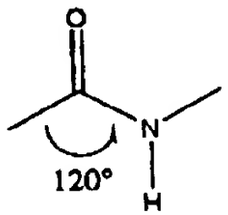
* corresponds to $M+K^+$

Table 4: Sequences and characteristics of the Leu^{28}-mutated β peptide analogues of Mart-1 27-35

	Sequences P1 P2 P3 P4 P5 P6 P7 P8 P9	Rt	mass
Mart-1 27-35 Leu^{28}	H- A -L - G- I - G - I - L- T - V -OH	14.20	857.0
b1	H-b-homoA-L- G - I - G - I - L- T - V -OH	14.47	871.2
b2	H- A-b-homoL- G - I - G - I - L- T - V -OH	14.16	870.9
b3	H- A - L -b-homoG-I - G - I - L- T - V -OH	14.07	871.0
b4	H- A -L - G -b-homoI-G - I - L- T - V -OH	14.20	871.4
b5	H- A -L - G - I -b-homoG-I - L- T - V -OH	14.14	871.3
b6	H- A -L - G - I - G -b-homoI-L - T - V -OH	14.05	871.7
b7	H- A -L - G - I - G - I -b-homoL-T - V -OH	13.78	871.3
b8	H- A -L - G - I - G - I - L -b-homoT-V -OH	13.56	871.5
b9	H- A -L - G- I - G - I - L- T -b-homoV-OH	14.14	871.4

3) Synthesis of the peptide [$Ile^{30}\psi(CH_2CH_2)Gly^{31}$] Mart-1 27-35

This analogue corresponds to the introduction of a carba bond in place of the peptide bond between the residues Ile^{30} and Gly^{31} of the antigen Mart-1 27-35.



peptide bond

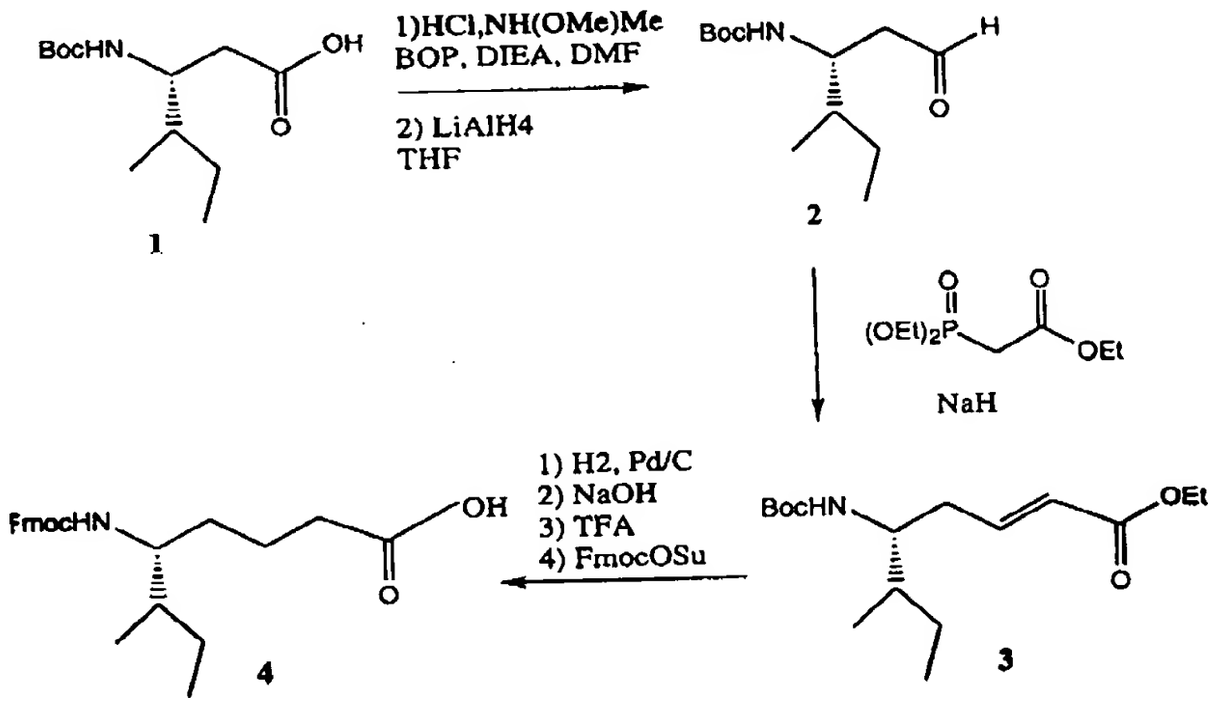
carba bond

Scheme 6: Schematic representation of peptide bonds and carba bonds

The carba bond gives the pseudopeptide a certain amount of flexibility, on account of the free rotations around the carbon-carbon bonds. These free rotations can result in changes in the orientation of the side chains (borne by the carbons Cα and Cα+1, see scheme 6) of the two amino acids located on either side of the carba bond. These changes in the orientation of the side chains can have many implications in the phenomena of binding, recognition and induction of various signals relative to those generated with the parent peptide.

The synthon Fmoc-Ileψ(CH_2CH_2)Gly-OH (4 in Figure 7) is necessary for the synthesis of the analogue [Ile^{30}ψ(CH_2CH_2)Gly^{31}] Mart-1 27-35. This synthon is obtained by chemical synthesis in several steps (scheme 7).

The dimethylhydroxamate of Boc-β-homoIle-OH (1) (see the preceding paragraph for the synthesis of the β-homo amino acids) is reduced with lithium aluminium hydride at low temperature (-25°C) in tetrahydrofuran in order to give the corresponding aldehyde (2). The Horner-Emmons reaction in the presence of triethyl phosphonoacetate gives the ethylenic dipeptide 3. Hydrogenation of the double bond, saponification of the ester, and deprotection and reprotection of the amine function with the Fmoc group allow the Fmoc-Ile ψ(CH_2CH_2)Gly-OH (4) to be obtained.



Scheme 7: Scheme of synthesis of Fmoc-Ile^{30} ψ(CH_2CH_2)Gly^{31}-OH (4)

This synthon (4) is incorporated in the solid phase in Fmoc chemistry. Coupling of 5 equivalents of the mixture (synthon/BOP/HOBt) is carried out twice in succession (20 min each time) in DMF. The Fmoc group is deprotected with 50% piperidine in DMF. The following amino acids are coupled conventionally.

The peptide is deprotected and detached from the resin in TFA, precipitated in ether and purified by RP-HPLC.

The pseudopeptide [Ile^{30} ψ(CH_2CH_2)Gly^{31}] Mart-1 27-35 is obtained in a purity of 100%. Its retention time in a gradient of from 5 to 65% B (with A = water containing 0.1% TFA and B=ACN containing 0.08% TFA is 13.49 min. Analysis of the mass of the pseudopeptide by MALDI with the aid of a Bruker Protein TOF spectrometer gives the expected mass M+H$^+$ 800.0.

4) Synthesis of the carba pseudopeptide analogues 1 to 8 of MART1 27-35

The synthesis of these peptides is carried out according to the procedure described in Rodriguez et al., Tetrahedron Letters, 1990, 31, 7319-7322.

The Mart-1 27-35 carba pseudopeptide 1 is obtained in a purity of 90%. Its retention time in a gradient of from 5 to 65% B (with the eluent : A = water containing 0.1% TFA and B = acetonitrile (ACN) containing 0.08% TFA) is 12.98/13.10 min. The analysis of the mass of the pseudopeptide by MALDI using a Bruker Protein TOF spectrometer gives the expected mass M+H$^+$ 799.1.

The Mart-1 27-35 carba pseudopeptide 2 is obtained in a purity of 98%. Its retention time in a gradient of from 5 to 65% B (with the eluent : A = water containing 0.1% TFA and B = acetonitrile (ACN) containing 0.08% TFA) is 12.90 min. The analysis of the mass of the pseudopeptide by MALDI using a Bruker Protein TOF spectrometer gives the expected mass M+H$^+$ 799.16.

The Mart-1 27-35 carba pseudopeptide 3 is obtained in a purity of 96% (3a), 95% (3b). Its retention time in a gradient of from 5 to 65% B (with the eluent : A = water containing 0.1% TFA and B = acetonitrile (ACN) containing 0.08% TFA) is 13.07 (3a), 13.54 (3b) min. The analysis of the mass of the pseudopeptide by MALDI using a Bruker Protein TOF spectrometer gives the expected mass M+H$^+$ 799.13.

The Mart-1 27-35 carba pseudopeptide 4 is obtained in a purity of 100%. Its retention time in a gradient of from 5 to 65% B (with the eluent : A = water containing 0.1% TFA and B = acetonitrile (ACN) containing 0.08% TFA) is 13.49 min. The analysis of the mass of the pseudopeptide by MALDI using a Bruker Protein TOF spectrometer gives the expected mass M+H$^+$ 800.03.

The Mart-1 27-35 carba pseudopeptide 5 is obtained in a purity of 99.7% (5a), 96% (5b). Its retention time in a gradient of from 5 to 65% B (with the eluent : A = water containing 0.1% TFA and B = acetonitrile (ACN) containing 0.08% TFA) is 12.72 (5a), 14.16 (5b) min. The analysis of the mass of the pseudopeptide by MALDI using a Bruker Protein TOF spectrometer gives the expected mass M+H$^+$ 799.1.

The Mart-1 27-35 carba pseudopeptide 6 is obtained in a purity of 86% (6a), 90% (6b). Its retention time in a gradient of from 5 to 65% B (with the eluent : A = water containing 0.1% TFA and B = acetonitrile (ACN) containing 0.08% TFA) is 13.25 (6a), 13.72 (6b) min. The analysis of the mass of the pseudopeptide by MALDI using a Bruker Protein TOF spectrometer gives the expected mass $M+H^+$ 799.16.

The Mart-1 27 carba pseudopeptide 7 has a retention time in a gradient of from 5 to 65% B (with the eluent : A = water containing 0.1% TFA and B = acetonitrile (ACN) containing 0.08% TFA) of 10.20 (7a), 13.61 (7b) min.

The Mart-1 27-35 carba pseudopeptide 8 is obtained in a purity of 99%. Its retention time in a gradient of from 5 to 65% B (with the eluent : A = water containing 0.1% TFA and B = acetonitrile (ACN) containing 0.08% TFA) is 15.24/15.29 min. The analysis of the mass of the pseudopeptide by MALDI using a Bruker Protein TOF spectrometer gives the expected mass $M+H^+$ 798.

B.2.) Synthesis of peptide analogues comprising non-protein-generating amino acids :

Synthesis of the peptide analogue [Tic62] M58-66

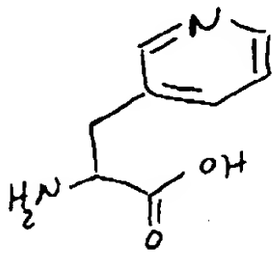
Tetrahydroisoquinoline-3-carboxylic acid is a constrained analogue of phenylalanine. It was introduced into position 62 in the sequence of the influenza matrix peptide in place of the natural phenylalanine in order to study the importance of the orientation of the phenyl group in the interaction with the MHC molecule and TCR.

The peptide analogue was synthesized by standard Fmoc chemistry. The derivative Fmoc-L-Tic-OH (sold by the company Néosystem) was introduced into the growing chain using a conventional coupling process (BOP/HOBt/Fmoc-L-Tic-OH) in a five-fold excess for 20 min in DMF. The deprotection of the Fmoc group was carried out in several steps with 50% piperidine in DMF (4 treatments of 30 min each, separated by 3 washes of the resin with DMF). Assembly of the following amino acids posed no particular problems. After standard deprotection with TFA and HPLC purification, the peptide was obtained in a purity of 90%. . The mass measured using a Bruker Protein TOF spectrometer was the expected mass $M+H^+$ 978.9.

The synthesis of the peptide analogue [Tic64]M58-66 is carried out in the same way as previously, introducing tetrahydroisoquinoline-3-carboxylic acid in place of the phenylalanine in position 64.

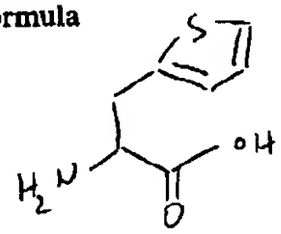
Still according to the same protocol :

. the analogues 3-Pya62 and 3-Pya64 of M58-66 were obtained by introducing the amino acid of the formula



in place of the phenylalanine in positions 62 and 64, respectively ;

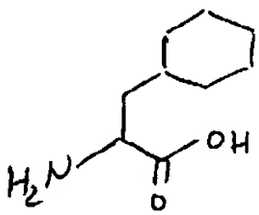
. the analogues 2-Tha62 and 2-Tha64 of M58-66 were obtained by introducing the amino acid of the formula



in place of the phenylalanine in positions 62 and 64, respectively ;

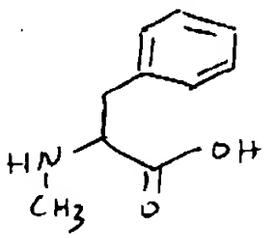
. the analogues D-Phe62 and D-Phe64 of M58-66 were obtained by introducing D-phenylalanine in place of the phenylalanine in positions 62 and 64, respectively ;

. the analogues Cha62 and Cha64 of M58-66 were obtained by introducing the amino acid of the formula



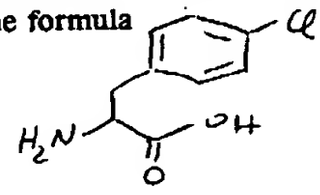
in place of the phenylalanine in positions 62 and 64, respectively ;

. the analogue N-MePhe62 of M58-66 was obtained by introducing the amino acid of the formula



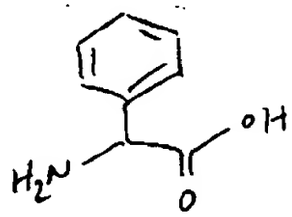
in place of the phenylalanine in position 62 ;

. the analogues pCl-Phe62 and pCl-Phe64 of M58-66 were obtained by introducing the amino acid of the formula



in place of the phenylalanine in positions 62 and 64, respectively ;

. the analogues Phg62 and Phg64 of M58-66 were obtained by introducing the amino acid of the formula



in place of the phenylalanine in positions 62 and 64, respectively.

II- Study of the biological properties of the peptide analogues synthesized:

A) Methodology :

1) Detection of the association between the peptides and the histocompatibility molecules

1.1. Equipment

Sources of histocompatibility molecules

They are currently of two main types : mutant cells and purified histocompatibility molecules.

- The mutant cell used is the human T2 cell (DeMars *et al.*, 1985; Ljunggren & Kärre, 1985 ; Salter & Cresswell, 1986) which is a variant of the T1 line produced by fusion of the CEM T lymphoma and of the B lymphoma 721,174. This cell, which lacks peptide carriers, contains heavy chains of molecules of category I free of peptides which will accept exogenous peptides.

- Histocompatibility molecules of category I purified by affinity chromatography from human B cell lines transformed with EBV can also be used. In this case, the endogenous peptides must be removed by means of a treatment with 1.5 M urea and 12.5 mM sodium hydroxide (pH 11.7) for 1 h at 4°C, followed by removing them by means of a desalination column (PD10, Pharmacia). The histocompatibility molecules are immediately put back in the presence of the peptides to be tested in a PBS buffer with 0.05 % Tween 20, 2 mM EDTA, 0.1% NP40 and 6 mM CHAPS, in the presence of 2 µg/ml B2m to facilitate the reassociation (Gnjatic *et al.*,1995).

Peptides

The peptides tested are used at concentrations ranging from 100 μM to 0.1 nM.

1.2. Assembly protocol

Aliquots of 8 x 10^5 T2 cells in a volume of 64 μl, distributed in Eppendorf microfuge tubes, are placed in the presence of a lysis buffer containing 10 mM PBS, pH 7.5, 1% NP40, protease inhibitors (1 mM PMSF, 100 μM iodoacetamide, 2 μg/ml aprotinin, 10 μM leupeptin, 10 μM pepstatin and 10 μg/ml trypsin inhibitor). The lysis is carried out in the presence of the peptides to be tested for 30 min or 1 h at 37°C. After elimination of the undissolved material by centrifugation at 15 000 rpm at 4°C, 140 μl of PBS containing 0.05% of Tween 20, 3 mM of sodium azide, 1 mM PMSF and 10 mg/ml of bovine albumin are added to the supernatant. Each sample is incubated for 20 h at 4°C in 2 wells of a Nunc or Maxisorb type microtitration plate precoated with a monoclonal antibody (Parham & Brodsky, 1981) (10 μg/ml in PBS) which recognizes the histocompatibility molecules having one (or more) conformation(s) which comply (complies)for the presentation of the peptides and which is (are) similar to that (those) present at the surface of the cells. The plate coated with antibody is presaturated with bovine albumin at 10 mg/ml in PBS-Tween before adding the sample. The second antibody which allows detection of the assembly of the histocompatibility molecules is directed against beta2m. It is coupled either with biotin (NHS-LC biotin, Pierce) or to alkaline phosphatase (P-5521, Sigma) and is incubated at 2 μg/ml for 1 h at 37°C. In the case of the use of biotin, an incubation for 45 min at 20-25°C with extravidin coupled with alkaline phosphatase (E-2636, Sigma) is carried out. The activity of the alkaline phosphatase is measured using, as substrate, 4-methyl-umbelliferyl phosphate (M-8883, Sigma) at 100 μM in 50 mM diethanolamine, pH 9.5 with 1 mM $MgCl_2$. The reading is carried out at 340/460 nm using a Millipore 2300 cytofluorimeter.

1.3. Controlling the stability of the HLA/peptide complexes

The material used is either purified HLA or the lysate of the T2 cell. With purified HLA, the endogenous proteins need to be removed, as described in paragraph I-1, and the HLA placed in the presence of the peptide to be tested in an Eppendorf tube at 37°C for times ranging from a few minutes to several days. The subsequent phase of incubation on a 96-well plate (see paragraph I-2) with the anti-HLA antibody (Parham & Brodsky, 1981) is carried out for 1 h at 37°C. The detection is standard (Teillaud *et al.*, 1982). With the T2 cell lysate, all the

incubations are also carried out at 37°C after all the protease inhibitors have been added.

1.4. Analysis of the conformation of the HLA/peptide complexes by BIAcore surface plasmonic resonance

This machine measures, with the aid of a reflected light beam, the molecular interactions which take place in real time in a continuous flow of buffer. The anti-HLA antibodies are diluted to 25 µg/ml in pH 7.4 sodium acetate buffer and are covalently bound via their free amines to the carboxymethyl dextran matrix of the "Sensor Chip CM5" activated with 50 mM NHS and 200 mM EDC. The injection is carried out at a rate of 5 µl/min for 6 minutes and the residual reactive groups are then blocked with 30 µl of pH 8.5 ethanolamine chloride. The HLA/peptide complexes formed as indicated in paragraph I-1 are injected (30 µl) at a rate of 2 µl/min and will be able to bind to the immobilized antibody. The results are expressed in resonance units (RU) which correspond to the angle of deflection of the light beam, this deflection being proportional to the concentration of proteins in contact with the matrix. The system can be reused after regeneration by injecting 1 M ethanolamine for 2 min. Kinetic and affinity measurements can be carried out.

2) Induction of cytolytic T lymphocytes *in vitro*

Unpurified peripheral blood mononuclear cells (PBMC) are cultured in RPMI 1640 and 10% BSA with antibiotics, at a concentration of 2x 10^6 /ml, at a rate of 2 ml per well (Costar 24-well plates). 1 µg/ml tetanus toxin (TT) is added to these cells to stimulate the CD4+ T cells, and the peptide to be tested is added as a CTL inducer at a rate of 1µg/ml. On day 3 or 4, 50 U/ml of IL-7 are added. For each culture, 10 replicates are treated independently. The effector cells generated are restimulated with PBMCs preincubated with the peptide (50 µg/ml for 4 h for 10x 10^6 cells) and then irradiated at 6000 rads. The stimulator cells are diluted to 106/ml and 1 ml is added per culture well after 1 ml of supernatant has been removed. The following day and 4 days later, IL-2 (10 U/ml) and IL-7 (50 U/ml) are added. The effectors are restimulated every 7 to 10 days in the same way. When the cytolytic activity appears, IL-2 is added at a rate of 50 U/ml. The cytotoxicity can be tested after 2 stimulations and then each week.

3) Analysis of the effector functions

3.1. Test of direct cytolysis

The induced T lines are tested as regards their capacity to recognize the inducer peptide presented on the HLA at the surface of the target cells, which are usually EBV lymphoblastoid cells. The cytolysis resulting therefrom is determined by the standard test of 4 h of release of ^{51}Cr. On day -3 or -4 before the test, the addition of interleukins is not carried out since the cells need to be weaned in order to be in optimum activation condition. For the primary inductions, the lysis cannot be detected before 3 weeks. The target cells labelled for 1 h with 10 μCi of ^{51}Cr are preincubated with the peptide (5 μg/ml conventionally or a variable concentration). After 2 washes, these cells are distributed in the wells of a 96-well microtitration plate at a rate of 5000 per well and the effector cells are added in ratios ranging from 1 to 100. The release of ^{51}Cr (R) obtained during the incubation of 4 h at 37°C is measured. The percentage of lysis is determined by the formula below:

(R. experimental - R. spontaneous / R. total - R. spontaneous) x 100

3.2. Analysis of the interleukins

Analysis of the expression of the genes coding for the interleukins is carried out by means of a semi-quantitative RT-PCR technique. PBMCs or purified CD8+ cells (1 to 2 x 10^6) are cultured in 24-well plates and stimulated either with anti-CD3+ monoclonal antibodies in the presence of 100 nM Phorbol Myristate Acetate (PMA) with autologous PBMCs which have been irradiated and sensitized or otherwise for 1 h 30 min with a peptide whose recognition is restricted by the molecules of the MHC. For the various interleukins, after 12 to 14 h of stimulation (or after 3, 10 and 24 h for the kinetic analysis of the appearance of the mRNAs), the cells are recovered and the total RNA is extracted by the technique with RNAzol (Bioprobe, Montreuil, France). The RNA (1 to 2 mg) is retrotranscripted with superscript II (Gibco BRL). The cDNAs synthesized are diluted to 1/5 with water and their concentration is quantified by competitive PCR with a plasmid (pQB.2) containing the sequence of B actin (237-bp product, generously supplied by D. Shire). The PCR reaction is carried out in the presence of 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (dATP, dCTP, dGTP, dTTP), 0.4 mM of sense and antisense primer, and 2 U of Taq polymerase (Promega). The amplification is carried out starting with a denaturation at 94°C for 5 min, followed by 30 cycles at 94°C, 30 s, 55°C, 30 s, 72°C, 30 s, and a final elongation at 72°C, 10 min, in a regulated thermostat (Perkin Elmers 9600). The analysis of IL-2, IL-4 and IFN-g is carried out under the same conditions as for B-actin, except that the number

of cycles ranges between 30 and 40. The relative quantification is carried out by competitive PCR.

B) Results

1) Peptide analogues of the peptide M58-66 containing non-protein-generating amino acids :

1.1. Binding to the A2 molecule of the peptides of the series M58-66 :

The results are represented in Figure 1 (Figures 1A to 1C).

The peptides were incubated at different concentrations at 4°C for 18 h in the presence of denatured HLA-A2 molecules and β2 microglobulin. The stable HLA-A2/peptide complexes formed at 4°C are captured with the antibody BB7.2 adsorbed onto a plate and are revealed with a second anti β2 microglobulin antibody coupled to alkaline phosphatase. The quantity of HLA-A2/peptide complexes formed is proportional to the intensity of the fluorescence detected with cytofluor. NP 383-391 is a negative control peptide. 0. peptide corresponds to the background noise in the absence of peptide. F.U. : arbitrary fluorescence unit.

Only two modifications affect the binding of these modified peptides to the molecule HLA-A2 : modification by the D-Phenyl in position 62 ([D-Phe62]M58-66) and in position 64 ([D-Phe64]M58-66).

1.2. Capacity of these peptides to induce lysis of the target T2 cells by means of a line of cytotoxic T cells (CTL) which is specific for M58-66 and restricted with the molecule A-A2.1.

The results are represented in Figure 2 (Figures 2A to 2D).

The target T2 cells A2 were treated with chromium (Cr51) and then incubated for 2 h with 1 μg/ml of peptide. After washing, the target cells (5000 per well) were incubated for 4 h with the CTL line at a ratio of 10 effector cells (E)/1 target cell (C). The specific lysis is expressed as a percentage of lysis as a function of the concentration of peptide incubated with the targets.

The modifications Phg-62 and 64, DPhe-62 and 64, Cha62 and 64, Tic 62 and 64 have an effect on the recognition of the cytolytic T lymphocytes which are specific for the peptide M58-66.

1.3. Dose response.

The results are represented in Figure 3 (Figures 3A and 3B).

The target T2 cells A2 were treated with (Cr51) and then incubated for 1 h with various concentrations of peptide. After washing, the target cells (5000 per well) were incubated for 4 h with the CTL line at a ratio 10E/1C. The specific

lysis is expressed as a percentage of lysis as a function of the concentration of peptide incubated with the targets.

The cytolytic T lymphocytes which are specific for the peptide M58-66 have a weaker affinity for the modified peptides.

2) Peptide analogues of Mart-1 comprising a bond of the β-homologation type :

2.1. Binding to the molecule A2.

The results are represented in Figure 4 (Figures 4A and 4B).

The various peptides are compared as regards their ability to bind to the purified HLA-A2.1 molecule.

The peptides were incubated at different concentrations at 4°C for 18 h in the presence of denatured HLA-A2 molecules and β2 microglobulin. The stable complexes HLA-A2/peptide formed at 4°C are captured with the antibody BB7.2 adsorbed onto a plate and revealed with a second anti β2 microglobulin antibody coupled with alkaline phosphatase. The quantity of HLA-A2/peptide complexes formed is proportional to the intensity of the fluorescence detected with cytofluor (F.U.: arbitrary fluorescence unit).

NP is an influenza virus nucleoprotein peptide which does not bind to the molecule HLA-A2 (negative control).

Apart from the β4 homo peptide which binds better than the parent peptide, the other β homo peptides have an intermediate to weak affinity relative to the peptide Mart-1.

2.2. Ability of these peptides to induce lysis of the target T2 cells with 3 T clones specific for Mart-1 which are restricted with the molecule HLA-A2.1.

The results are represented in Figure 5.

The target T2 cells A2 were treated with chromium (Cr51) and then incubated for 2 h with 1 μg/ml of peptide. After washing, the targets (1500 per well) were incubated for 4 h with the various T clones (LT8, LT11, LT12) at a ratio of 3 effector cells/1 target cell (E/T). The specific lysis is expressed as a percentage of lysis.

The target T2 cells A2 incubated with the parent peptide Mart-1 are 90% lysed with the three cytolytic clones (LT8, LT11 and LT12) which are specific for this peptide. In the absence of peptide (0), no lysis is detected. Only the β4 homo peptide is capable of inducing a lysis activity similar to that of the parent peptide for only one clone (LT12).

2.3. Dose response.

The results are represented in Figure 6.

The target T2 cells A2 were treated with chromium (Cr51) and then incubated for 2 h with various concentrations of peptide. After washing, the targets (1500 per well) were incubated for 4 h with the T clone LT12 at a ratio 3E/1C. The specific lysis is expressed as a percentage of lysis as a function of the concentration of peptide incubated with the targets.

The T clone LT12 has a weaker affinity for the β4 peptide than for the peptide Mart-1.

3) Peptide analogues of the peptide Mart-1 containing a -CH_2-NH- bond.

3.1. Binding of the peptides of the reduced Mart-1 series to the molecule A2.

The results are represented in Figure 7 (Figures 7A and 7B).

Peptide/HLA-A2 complexes formed as a function of the concentration of peptide :

The peptides were incubated at two different concentrations (10^{-4} M and 10^{-6} M) at 4°C for 18 h in the presence of denatured HLA-A2 molecules and β2 microglobulin. The stable HLA-A2/peptide complexes formed at 4°C are captured with the antibody BB7.2 (which is specific for this molecule of category I and is adsorbed at the bottom of the wells) and revealed by a second anti β2 microglobulin antibody coupled with alkaline phosphatase. The quantity of HLA-A2/peptide complexes formed is proportional to the intensity of the fluorescence detected with cytofluor (F.U. : arbitrary fluorescence unit).

The peptides ψ 1-2 to 4-5 have an intermediate to weak affinity for the peptide Mart-1. The peptides ψ 4-5 to 8-9 bind very weakly to the purified A2.1 molecule.

3.2. Ability of the peptides of the reduced Mart series to induce lysis of the target T2 cells with 3 T clones specific for Mart which are restricted with the molecule HLA-A2.1.

The results are represented in Figure 8.

Lysis test :

Target T2 cells A2 were treated with chromium (Cr51) and then incubated for 2 h with 1 μg/ml of peptide. After washing, the targets (1500 per well) were incubated for 4 h with the various T clones (LT8, LT11, LT12) at a ratio of 3 effector cells/1 target cell (E/T). The specific lysis is expressed as a percentage of lysis as a function of the various clones tested.

The target T2 cells incubated with the parent peptide Mart-1 are 90% lysed with the three cytolytic clones (LT8, LT11 and LT12) which are specific for this peptide. In the absence of peptide (0), no lysis is detected. Only the peptides ψ 2-3 and ψ 5-6 are capable of inducing a lysis activity similar to that of the parent peptide for a single clone (LT8 and LT12 respectively). ψ 7-8 induces a weaker lysis activity, detected only with the clone LT12.

3.3. Dose response of the peptides which induce a lysis with the clones LT8 and LT12.

The results are represented in Figure 9 (Figures 9A and 9B).

Lysis test :

Target T2 cells A2 were treated with chromium (Cr51) and then incubated for 2 h with various concentrations of peptide. After washing, the targets (1500 per well) were incubated for 4 h with the various T clones (LT8, LT12) at a ratio of 3E/1C. The specific lysis is expressed as a percentage of lysis as a function of the concentration of peptide incubated with the targets.

The peptides ψ 2-3 and ψ 5-6 have a dose response which is comparable to that of the parent peptide, whereas the peptide ψ 7-8 only induces lysis weakly, even at a high concentration of peptide. This result suggests the modification introduced reduces the affinity of the TCR (T receptor) of the clone LT8 for this peptide.

4) Peptide analogues of the peptide M58-66 containing a -CH_2-NH- bond.

4.1. Binding of the peptides of the reduced M58-66 series to the molecule A2.

The results are represented in Figure 10 (Figures 10A and 10B).

The peptides were incubated at various concentrations at 4°C for 18 h in the presence of denatured HLA-A2 molecules and β2 microglobulin. The stable HLA-A2/peptide complexes formed at 4°C are captured with the antibody BB7.2 adsorbed on the plate and are revealed with a second anti β2 microglobulin antibody coupled with alkaline phosphatase. The quantity of HLA-A2/peptide complexes formed is proportional to the intensity of the fluorescence detected with cytofluor. F.U. : arbitrary fluorescence unit.

The peptides Mart-1 modified by reduction have an intermediate affinity for the molecule HLA-A2. The reduction in positions 2-3 and 8-9 makes the binding undetectable for the clone studied.

4.2. Ability of the peptides of the reduced M58-66 series to induce lysis of the target T2 cells with a CTL line induced with M58-66 and restricted with the molecule HLA-A2.1.

The results are represented in Figure 11.

The target T2 cells A2 were treated with chromium (Cr51) and then incubated for 1 h with 1 μg/ml of peptide. After washing, the targets (1500 per cell) were incubated for 4 h with the CTL line at a ratio of 10E/1C. The specific lysis is represented as a percentage of lysis.

The reduction in positions 2-3 and 5-6 does not have an effect on the recognition of the peptide by the cytolytic T lymphocytes which are specific for the peptide M58-66.

KEY TO THE FIGURES :

- Figure 1 : study of the binding of the peptide analogues of the peptide M58-66 containing non-protein-generating amino acids to the molecules of the MHC (HLA-A2) : the concentrations of the peptide analogues (10^{-4} to 10^{-10} M) are indicated on the x-axis and the fluorescence units are indicated on the y-axis :

. Figure 1A : study of the binding of the peptide analogues [Tic62]M58-66 (also known as M58-66 Tic62 or Tic62), [3-Pya62]M58-66 (3-Pya62), [2-Tha62]M58-66 (2-Tha62), [D-Phe62]M58-66 (D-Phe62), [Tic64]M58-66 (Tic64),

. Figure 1B : study of the binding of the peptide analogues [3-Pya64]M58-66 (3-Pya64), [2-Tha64]M58-66 (2-Tha64), [D-Phe64]M58-66 (D-Phe64), [Cha62]M58-66 (Cha62), [N-MePhe62]M58-66 (N-MePhe62),

. Figure 1C : binding of the peptide analogues [pCl-Ph62]M58-66 (pCl-Phe62), [Phg62]M58-66 (Phg62), [Cha64]M58-66 (Cha64), [pCl-Phe64]M58-66 (pCl-Phe64), [Phg64]M58-66 (Phg64),

in comparison with the peptide M58-66, the negative control peptide NP383-391, and a solution containing no peptide (0 peptide).

- Figure 2 : study of the effect of peptide analogues of M58-66 containing non-protein-generating amino acids on the lysis of a line of target T2 cells with a line of cytotoxic T cells which are specific for M58-66 : the percentage of lysis is indicated on the y-axis and the ratio E/T (effector cells/target cells) is represented on the x-axis:

Figure 2A : effect of the analogues Tic62 and Tic64 on the lysis of the T2 cells,

Figure 2B : effect of the analogues D-Phe62, D-Phe64, Cha62 and Cha64 on the lysis of the T2 cells,

. Figure 2C : effect of the analogues 3-Pya62, 3-Pya64, 2-Tha62 and 2-Tha64 on the lysis of the T2 cells,

. Figure 2D : effect of the analogues pCl-Phe64, Phg64, pCl-Phe62 and Phg62 on the lysis of the T2 cells,

in comparison with the effects of the peptide M58-66 and of a solution containing no peptide (0 peptide).

- Figure 3 : study of the response of cytotoxic T cells as a function of the concentration of peptide analogues of the peptide M58-66 containing non-protein-generating amino acids : the percentage of lysis is indicated on the y-axis and the peptide concentration is indicated on the x-axis :

. Figure 3A : study as a function of the concentration of pCl-Phe62 and pCl-Phe64,

. Figure 3B : study as a function of the concentration of 2-Tha62 and 2-Tha64,

in comparison with the concentration of M58-66.

- Figure 4 : study of the binding of the peptide analogues of the peptide Mart-1 containing a bond of the β-homologation type to the molecules of the MHC (HLA-A2); the concentrations of the peptide analogues (10^{-5} to 10^{-7} M) are indicated on the x-axis and the fluorescence units are indicated on the y-axis :

. Figure 4A : study of the binding of the analogues β1 homo (B1), β2 homo (B2), β3 homo (B3) and β4 homo (B4),

. Figure 4B : study of the binding of the analogues β5 homo (B5), β6 homo (B6), β7 homo (B7), β8 homo (B8) and β9 homo (B9),

in comparison with the peptide Mart-1 (Mart) and a negative control peptide (NP).

- Figure 5 : study of the effect of peptide analogues of the peptide Mart-1 containing a bond of the β-homologation type on the lysis of target T2 cells with 3 clones of cytotoxic T cells (LT8, LT11 and LT12) which are specific for Mart-1: the percentage of lysis is indicated on the y-axis and the peptide analogues B1 to B9 are indicated on the x-axis, in comparison with Mart-1 and a solution without peptide (0).

- Figure 6 : study of the response of the cytotoxic T cells as a function of the concentration of the β4 homo analogue of Mart-1, in comparison with Mart-1 : the percentage of lysis is indicated on the y-axis and the concentration of peptide (μg/ml) is indicated on the x-axis.

- Figure 7 : study of the binding of the peptide analogues of the peptide Mart-1 containing a $-CH_2-NH-$ bond (reduced analogues) to the molecules of the MHC (HLA-A2) : the peptides at concentrations of 10^{-6} M or 10^{-4} M are indicated on the x-axis and the fluorescence units are indicated on the y-axis :

. Figure 7A : study of the binding of the peptides ψ(1-2)Mart-1 (represented by 1-2), ψ(2-3)Mart-1 (2-3), ψ(3-4)Mart-1 (3-4) and ψ(4-5)Mart-1 (4-5),

. Figure 7B : study of the binding of the peptides ψ(5-6)Mart-1 (5-6), ψ(6-7)Mart-1 (6-7), ψ(7-8)Mart-1 (7-8) and ψ(8-9)Mart-1 (8-9), in comparaison with Mart-1.

- Figure 8 : study of the effect of the reduced analogues 1-2 to 8-9 of Mart-1 on the lysis of T2 cells with 3 clones of T cells (LT8, LT11 and LT12) which are specific for Mart-1 : the percentage of lysis is indicated on the y-axis and the peptide analogues 1-2 to 8-9 are indicated on the x-axis, in comparison with Mart-1 and a solution without peptide (0).

- Figure 9 : study of the response of the cytotoxic T cells as a function of the concentration of reduced analogues of Mart-1: the percentage of lysis is indicated on the y-axis and the peptide concentration is indicated on the x-axis (μg/ml) :

. Figure 9A : study as a function of the concentration of the analogues 2-3 and 7-8,

. Figure 9B : : study as a function of the concentration of the analogue 5-6, in comparison with the concentration of Mart-1.

- Figure 10 : study of the binding of peptide analogues of the peptide M58-66 containing a $-CH_2-NH-$ bond (reduced analogues) to the molecules of the MHC HLA-A2) : the peptide concentrations (10^{-10} to 10^{-4} M) are indicated on the x-axis and the fluorescence units are indicated on the y-axis :

. Figure 10A : study of the binding of the peptides ψ(1-2)M58-66 (1-2), ψ(2-3)M58-66 (2-3), ψ(3-4)M58-66 (3-4) and ψ(4-5)M58-66 (4-5),

. Figure 10B : study of the binding of the peptides ψ(5-6)M58-66 (5-6), ψ(6-7)M58-66 (6-7), ψ(7-8)M58-66 (7-8) and ψ(8-9)M58-66 (8-9), in comparison with M58-66 and a mutated peptide (GIL) of M58-66 bearing the mutation GLL → GIL [M58-66 (GIL)],

- Figure 11 : study of the effect of the reduced analogues 1-2 to 8-9 of M58-66 on the lysis of T2 cells with a clone of T cells which is specific for M58-66 : the percentage of lysis is indicated on the y-axis and the effector cell/target cell (E/T) ratio is indicated on the x-axis, in comparison with M58-66, mutated (GLL) M58-66 and a solution without peptide (0).

BIBLIOGRAPHIC REFERENCES

1. Bohnstedt A.C. *et al.* Tetrahedron Lett. 1993, 34:5217-5220.

2. Breton P. *et al.* Int. J. Peptide Protein Res. 1990, 35:346-351.

3. Chorev M. & Goodman M. Acc. Chem.. Res. 1993, 26:266-273.

4. DeMars *et al.* Proc. Natl. Acad. Sci. USA 1985, 82:8183.

5. Evans B.E. *et al.* J. Org. Chem. 1985, 50:4615-4625.

6. Gnjatic S. *et al.* Eur. J. Immunol. 1995, 25:1638-1642.

7. Gonzalez-Muniz R. *et al.* J. Med. Chem. 1995, 38:1015-1021.

8. Goodman M. & Chorev M. Acc. Chem. Res. 1979, 12:1-7.

9. Harbeson S.L. & Rich D.H. J. Med. Chem. 1989, 32:1378-1392.

10. Hann M.M. *et al.* J. Chem. Soc. Perkin Trans. 1982, 1:307-314.

11. Kempf D.J. *et al.* Int. J. Peptide Protein Res. 1991, 38:237-241.

12. Ljunggren H.G. & Kärre K. J. Exp. Med. 1985, 162:1745.

13. Martinez J. *et al.* J. Med. Chem. 1985, 28:1874.

14. Parham P. & Brodsky F.M. J. Immunol. 1981, 3:277.

15. Rodriguez M. *et al.* Tetrahedron Lett. 1990a, 31:5153-5156.

16. Rodriguez M. *et al.* Tetrahedron Lett. 1990b, 31:7319-7322.

17. Salter R.D. & Cresswell P. EMBO J. 1986, 5:943.

18. Sasaki Y. & Coy D.H. Peptides 1987, 8:119-121.

19. Shemyakin M.M. *et al.* Angew Chem. Internat. Edit. 1969, 8:492-499.

20. Szelke M. *et al.* Nature 1982, 299:555-557.

21. Teillaud *et al.* Immunogenetics 1982, 15:377.

22. TenBrink R.E. J. Org. Chem. 1987, 52:418-422.

23. Wuts P.G.M. *et al.* J. Org. Chem. 1992, 57:6696-6700.